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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		Attorney Docket Number 045636-5025
		U.S. Application No. Unassigned 09/319724
International Application. No.	International Filing Date	Priority Date Claimed
PCT/EP97/07339	12 December 1997	12 December 1996

Title of Invention

MAMMALIAM ICYP (IODOCYANOPINDOLOL) RECEPTROR AND ITS APPLICATIONS

Applicants For DO/EO/US

Gerlinde LENZEN, Arthur Donny STROSBERG, Toshinari SUGASAWA and Shigeaki MOROOKA

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(l).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventors (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 14. below concern other document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [X] Other items or information:
    a. [X] WIPO Publication WO98/26065
    b. [X] International Search Report
    c. [X] PCT/RO/101
    d. [X] PCT/RO/105
    e. [X] PCT/IB/301
    f. [X] PCT/IB/304
    g. [X] PCT/IB/308
    h. [X] PCT/IPEA/409

U.S. APPLICATION NO.	INTERNATIONAL APPLICATION NO.	ATTORNEY DOCKET NUMBER
09/319724 Unassigned	PCT/EP97/07339	045636-5025

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15. [X] The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO or JPO.....$840.00
International preliminary examination fee paid to USPTO (37 CFR 1.482).....$670.00
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).....$760.00
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....$970.00
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....$96.00

ENTER APPROPRIATE BASIC FEE AMOUNT =				$ 840.00
Surcharge of $130.00 for furnishing the oath or declaration later than **[ ] 20 [ ] 30** months from the earliest claimed priority date (37 CFR 1.492(e)).				$
Claims	Number Filed	Number Extra	Rate	
Total Claims	25 - 20 =	5	X $18.00	$ 90.00
Independent Claims	1 - 3 =	0	X $78.00	$
Multiple dependent claim(s) (if applicable)			+$260.00	$
TOTAL OF ABOVE CALCULATIONS =				**$930.00**
Reduction by ½ for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28)				-$
SUBTOTAL =				**$930.00**
Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				+$
TOTAL NATIONAL FEE =				**$930.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property				+$
TOTAL FEES ENCLOSED =				**$930.00**
			Amount to be refunded	$
			charged	$

a. [X] A check in the amount of **$930.00** representing $840.00 filing fee and $90.00 additional claims fee is enclosed.

b. [ ] Please charge my Deposit Account No. 50-0310 in the amount of $-0- to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] **Except** for issue fees payable under 37 C.F.R. §1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 CFR §1.16 and §1.17 which may be required, or credit any overpayment to Deposit Account No. 50-0310.

Customer No. 009629
SEND ALL CORRESPONDENCE TO:
Morgan, Lewis & Bockius LLP
1800 M Street, N.W.
Washington, D.C. 20036
(202) 467-7000

Elizabeth C. Weimar
Elizabeth C. Weimar
Reg. No. P-44,478

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PATENT
ATTORNEY DOCKET NO.: 045636-5025

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Gerlinde LENZEN *et al.*

Application No.: Unassigned
(National Stage Application of
PCT/EP97/07339 filed December 12, 1997)

Filed: June 11, 1999

For: **MAMMALIAN ICYP (IODOCYANOPINDOLOL) RECEPTOR AND ITS APPLICATIONS**

Group Art Unit: Unassigned

Examiner: Unassigned

ATTN: BOX PCT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

PRELIMINARY AMENDMENT

Prior to the examination of the above-identified application on the merits, please amend the application as follows:

IN THE CLAIMS:

Please cancel claims 1-21 without prejudice or disclaimer of the subject matter contained therein and substitute therefor the following claims 22-46.

--22. Substantially pure mammal non-adrenergic receptor polypeptide characterized in that it contains sites such that when said sites are exposed at the surface of a cell, they are capable of binding iodocyanopindolol (ICYP) under blockade of α, β1, β2, β3-AR, serotonine 5-HT_{1A} and serotonine 5-HT_{1B} receptors, said binding being saturable, reversible, able to be

displaced by a β-adrenergic receptor agonist SM-11044 with stereoselectivity but not by isoproterenol, norepinephine, epinephrine, serotonine, dopamine or BRL-37344, and not being blocked by propranolol, said polypeptide (1) having an apparent molecular weight of about 30-40 kDa when labeled with ^{125}I-iodocyanopindolol after photoaffinity labeling and separation by electrophoresis and an apparent molecular weight of about 60-80 kDa in Western blot, and (2) generating a fragment having the following formula $DPX_1FFQHRIHX_2FSIFNX_3$ by acidic cleavage, wherein X_1 represents S (SEQ ID No. 5) or X (SEQ ID No. 6), X_2 represents V (SEQ ID No 6) or W (SEQ ID No. 5) and X_3 represents S (SEQ ID No. 5) or H (SEQ ID No. 6)

23. The polypeptide according to claim 22, characterized in that it contains at least SEQ ID No. 1.

24. The polypeptide according to claim 22, characterized in that it consists of SEQ ID No. 13.

25. An isolated and purified nucleic acid sequence, characterized in that it encodes a mammalian receptor as claimed in claim 22.

26. The isolated and purified nucleic acid sequence of claim 25, characterized in that it includes at least SEQ ID No. 2.

27. The isolated and purified nucleic acid sequence of claim 25, characterized in that it consists of SEQ ID No. 14.

28. The purified nucleic acid sequence according to claim 25, characterized in that it hybridizes with SEQ ID No. 3 or SEQ ID No. 4.

29. A cDNA clone, comprising an isolated and purified nucleic acid sequence according to claim 25.

30. A synthetic or non-synthetic nucleotide probe, characterized in that it hybridizes with a nucleic acid according to claim 25, or with its complementary sequence or its corresponding RNA, said probe being unable to hybridize with the genes or the messenger RNA coding for β-adrenergic receptors.

31. A probe according to claim 30, selected from the group consisting of SEQ ID No. 3, SEQ ID No. 4 and SEQ ID No. 7 to SEQ ID No. 12.

32. A primer for amplifying a nucleic acid sequence according to claim 25, selected from the group consisting of SEQ ID No. 7 to SEQ ID no. 12.

33. A recombinant plasmid for cloning and/or expression, containing a nucleic acid sequence according to claim 25, inserted in a cloning site which is non-essential for replication.

34. The recombinant plasmid according to claim 33, further comprising an origin of replication for replication in a host cell, at least one gene whose expression permits selection of said host cell transformed with said plasmid, and a regulatory sequence, including a promoter permitting expression of said nucleic acid sequence in said host cell.

35. The recombinant plasmid according to claim 33, comprising plasmid pcDNA3 into which is inserted, in a multisite linker, SEQ ID No. 2, wherein said recombinant plasmid is deposited as CNCM No. I-1795.

36. A host cell transformed by a recombinant plasmid according to claim 33, comprising the elements of regulation necessary for the expression of said nucleotide sequence in said host cell.

37. The host cell according to claim 36, characterized in that it is a mammalian cell line.

38. An antibody directed specifically against the receptor polypeptide of claim 22, which antibody fails to recognize either known α or β-adrenergic, or serotonine, or dopamine receptors.

39. A method for assaying a substance for agonist or antagonist activity towards a receptor polypeptide of claim 22, which method comprises:

- placing the substance in contact with tissue membrane proteins or a transformed host cell expressing said receptor polypeptide under conditions which permit binding between said polypeptide binding sites and an agonist or an antagonist thereto and
- measuring an appropriate transduction signal.

40. A process for studying the binding affinity of a compound for a receptor polypeptide of claim 22, which process comprises:

- transforming a host cell by an expression vector comprising a nucleotide sequence coding for said receptor polypeptide,

- culturing said transformed host cell under conditions which permit the expression of said receptor polypeptide encoded by said nucleotide sequence and the transfer of the expressed receptor polypeptide to the membrane of the said transformed host cell so that transmembrane sequences of said receptor polypeptide are embedded in the cell membranes of the transformed host cell;

- placing said transformed host cell in contact with said compound and

- measuring the quantity of said compound bound to said receptor polypeptide.

41. A process for studying the binding affinity of a compound for a receptor polypeptide of claim 22, which process comprises:

- extracting membrane proteins corresponding to said receptor polypeptide from appropriate tissue or cells,

- placing said membrane proteins in contact with said compound and

- measuring the quantity of said compound bound to said receptor polypeptide.

42. Method of labeling a receptor polypeptide of claim 22, which method comprises:

- extracting membrane proteins from a tissue containing said receptor polypeptide,

- labeling said membrane proteins with [^{125}I]-ICYP-diazirine or another appropriate marker under blockade of α, β1, β2, β3-AR and serotonine receptors,

- separating said labeled proteins by preparative SDS-PAGE electrophoresis and

- extracting the radioactive band.

43. A probe according to claim 31, further comprising a label.

44. A probe according to claim 43, wherein the label is a radioactive isotope, a detectable enzyme or a fluorochrome.

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45. A process according to claim 41, wherein the appropriate tissue or cells comprise muscle tissue or myocytes.

46. A method according to claim 42, wherein the tissue containing said receptor polypeptide comprises rat colon tissue or human skeletal muscle tissue.--

REMARKS

No new matter has been introduced by this Preliminary Amendment, which is being submitted to clarify the claims, and to remove the multiple dependency from the claims.

If there are any additional fees due in connection with the filing of this Preliminary Amendment, please charge the fees to our Deposit Account No. 50-0310.

Respectfully submitted

MORGAN, LEWIS & BOCKIUS LLP

By: Elizabeth C. Weimar
Elizabeth C. Weimar
Reg. No. P44,478

Dated: June 11, 1999

Customer No. 009629
MORGAN, LEWIS & BOCKIUS LLP
1800 M Street, N.W.
Washington, DC 20036
202-467-7000

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MAMMALIAN ICYP (IODOCYANOPINDOLOL) RECEPTOR AND ITS APPLICATIONS

The present invention relates to an isolated and substantially pure mammal polypeptide different from known adrenergic, serotonine and dopamine receptors, existing at least on mammalian muscle and eosinophils membranes, for instance in rat, guinea pig and humans.

The invention also relates:

to plasmids containing the genes coding for said polypeptide,

to host cells transformed by genes coding for the above mentioned polypeptide,

to nucleotide probes capable of hybridizing with the genes coding for the above mentioned polypeptide, and

to polyclonal and monoclonal antibodies directed against the above mentioned polypeptide and which can be used for the purpose of *in vitro* diagnosis,

A wide variety of membrane receptors for hormones and neurotransmitters are composed of a single polypeptide chain containing seven hydrophilic sequences and may be coupled to guanine-nucleotide-binding regulatory G proteins, which upon activation by agonists or antagonists, stimulate or inhibit various effectors such as enzymes or ion channels.

Among the family of seven transmembrane domains receptors are those for adrenaline and other catecholamines, the adrenergic receptors and those for acetylcholine and related muscarinic ligands, the muscarinic cholinergic receptors. Other similar proteins belonging to this growing family are those for serotonine, for dopamine, for tachykinins and for the pituitary glycoprotein hormones, to mention but a few.

The existence of atypical adrenergic receptors (AR), in adipocytes, in gastrointestinal tissues and in skeletal muscles has been well-established. Atypical β-adrenergic receptors (β-ARs) are defined as β-AR that can not be classified as typical β-ARs (β1-AR and β2-AR) with low β-AR antagonist effect, showing a propranolol (a classical non-selective β-AR antagonist)-resistant feature.

For instance, McLaughlin, MacDonald and co-workers characterized β-AR in rat colon (McLaughlin, MacDonald, 1990; MacDonald and Lamont, 1993; McKean and MacDonald, 1995). Propranolol was a weak antagonist against isoproterenol and BRL-37344. The propranolol-resistant responses of isoproterenol were antagonized by cyanopindolol with a pA_2 value of 7.12 under blockade of β1- and β2-AR effects. They reported that responses to isoproterenol in rat colon were mediated largely through β3-AR with small contribution of β1-AR and β2-AR (McKean and MacDonald, 1995). This observation is supported by Ek et al., 1986, who found β1- and β2-AR in rat colon membranes by [^{125}I]-pindolol binding studies. Thus, rat colon has mainly β3-AR in addition to β1- and β2-ARs. Like in guinea pig ileum, cyanopindolol acted as an antagonist at rat atypical β-AR, while it acted as a β1-, β2-AR antagonist having β3-AR agonist potency at human and mouse β3-AR (Blin et al., 1993).

Most of the pharmacological features of atypical β-ARs can be explained by β3-AR-activity; however, lack of β3-AR transcripts in skeletal muscles, or heterogeneous responses in vascular smooth muscles remained unexplained and show the complexity found in the field of receptors.

The invention solves an unresolved question with regard to the existence of polypeptide having a receptor activity other than that of β3-adrenergic receptors; in fact, it provides access to a novel receptor class present at least in muscles and in eosinophils, which displays transmembrane domains and may have signal transduction function.

The Inventors have now found, unexpectedly, that in rat colon smooth muscle membranes, there is a non-adrenergic, non-serotonine and non-dopamine receptor mediating at least inhibition of depolarized colon tonus.

The subject of the present invention is a substantially pure mammal polypeptide containing sites such that when said sites are exposed at the surface of a cell, they are able of binding iodocyanopindolol (ICYP) under blockade of α, β1, β2, β3-AR, serotonine 5-HT_{1A} and serotonine 5-HT_{1B} receptors, said binding being saturable, reversible, able to be displaced by a β-adrenergic receptor agonist SM-11044 with stereo-

selectivity but not by isoproterenol, norepinephrine, epinephrine, serotonine, dopamine or BRL-37344, and not being blocked by propanolol said polypeptide (1) having an apparent molecular weight of about 30-40 kDa when labeled with ^{125}I-iodocyanopindolol after photoaffinity labeling and separation by electrophoresis and an apparent molecular weight of about 60-80 kDa in Western blot, and (2) generating a fragment having the following formula $DPX_1FFQHRIHX_2FSIFNX_3$ by acidic cleavage, wherein, X_1 represents S (SEQ ID N°5) or X (SEQ ID N°6), X_2 represents V (SEQ ID N°6) or W (SEQ ID N°5) and X_3 represents S (SEQ ID N°5) or H (SEQ ID N°6), said polypeptide being present at least on muscles and eosinophils membranes and being a non-adrenergic receptor.

Said new non-adrenergic receptor has the following affinities with different β3-AR agonists and antagonists:

SM-11044 stimulates guinea pig ileum relaxation of KCl-induced tonus more efficiently than rat white adipocyte lipolysis; SM-11044 and BRL-35135A, a potent β3-AR agonist, display the additional property of inhibiting leukotriene B4 induced-guinea pig eosinophil chemotaxis, whereas isoproterenol and BRL-37344 had no such effect. This inhibition was unaffected by the non-selective β-AR antagonist, propranolol, but was antagonized by alprenolol, a β1-, β2-AR antagonist/β3-AR partial agonist.

While rat colon indeed contains β3-AR (Bensaid M. et al., 1993) in addition to β2-AR with a small population of β1-AR (Arunlakshana O. et al., 1959), the instant invention clearly shows the existence of a novel functional binding site in rat colon. This site was characterized by ligand binding and photoaffinity labeling, revealing a novel binding protein, designated here Ro-SMBP (SM-11044 binding protein or Rodent SM-binding protein).

Said new non-adrenergic SM-binding protein has also been found in human muscles (smooth and striated) (Hu-SMBP); it contains at least the sequence SEQ ID NO:1.

According to an advantageous embodiment of said protein it consists of SEQ ID NO:13.

Said protein contains a hydrophobic C-terminal region of 356 residues, which may contain up to nine transmembrane regions.

The invention also relates to an isolated and purified nucleic acid which encodes a mammalian receptor as hereabove defined and fragments thereof.

In humans, said coding sequence includes at least SEQ ID NO:2.

According to an advantageous embodiment of said coding sequence, it consists of SEQ ID NO:14, which corresponds to SMBP cDNA.

The said SEQ ID NO:14 comprises in particular the following single restriction sites: BstU I, Hha I, HinP I, Ava I, Sma I, Xma I, BsaA I, Apa I, Ban II, Bsp120 I, Eco0109 I, Sca I, Xmn I, Dra I, Nsi I, Ppu10 I, Acc65 I, Ban I, Kpn I, Bsp1407 I, Spe I, BspD I, Cla I, Hinf I, Tfi I, Avr II, Drd I, Esp3 I, Bpm I, PflM I, Bsm I, Alu I, BceF I, Bgl II, BstY I, ApaL I, Age I, BsrF I, Nsp I, Nsp7524 I, NspC I, as located in figures 19, 20 and 21.

This sequence encodes a polypeptide of 576 amino acid residues which contains a hydrophilic N-terminal region of 220 residues and a hydrophobic C-terminal region of 356 residues.

Said nucleic acid sequences in different mammals at least hybridizes with:

- a 900 bp of SEQ ID NO:3, or
- a 300 bp of SEQ ID NO:4.

Said fragments are useful for detection of the gene coding for the instant new non-adrenergic receptor.

The subject of the present invention is also cDNA clones, characterized in that they comprise a sequence fragment coding for the instant non-adrenergic receptor.

According to the invention, the clone designated 24.3 comprises 1,7 kb and includes SEQ ID NO:2; it encodes the instant Hu-SMBP.

The invention also relates to synthetic or non-synthetic nucleotide probes, characterized in that they hybridize with one of the nucleic acid as defined above or with their complementary sequences or their corresponding RNA, these probes being

such that they do not hybridize with the genes or the messenger RNA coding for β-adrenergic receptors.

Said probes are selected, for instance, from the group consisting of the hereabove mentioned 900 bp (SEQ ID NO:3) and 300 bp (SEQ ID NO:4) fragments and from SEQ ID NO:7 to SEQ ID NO:12, optionally labeled using a label such as a radioactive isotope, a suitable enzyme or a fluorochrome.

SEQ ID NO:7 to SEQ ID NO:12 may be used as primers for amplifying one of the instant nucleic acid sequence.

The hybridization conditions are defined as follows, for the probes possessing more than 100 nucleotides: 600 mM NaCl; 60 mM Na-citrate; 8 mM Tris-HCl pH 7,5; 50 mM Na-phosphate; 1% Ficoll; 1% polyvinylpyrrolidone; 1% bovine serum albumine; 40% formamide; 0.2% SDS; 50 µg/ml salmon sperm DNA.

The invention also relates to recombinant plasmid, cosmid or phage in particular for cloning and/or expression, containing a nucleic acid sequence of the invention at one of its cloning sites (non essential for its replication).

According to an advantageous embodiment of the said plasmid, it further comprises an origin of replication for replication in a host cell, at least one gene whose expression permits selection of said host cell transformed with said plasmid, and a regulatory sequence, including a promoter permitting expression of a polypeptide having a non-adrenergic activity as defined hereabove, in said host cell.

According to an advantageous arrangement of this embodiment, the said plasmid is pcDNA3 into which is inserted, in a multisite linker, SEQ ID NO:2, wherein said plasmid is deposited with the Collection Nationale de Cultures de Microorganismes [National Collection of Microorganism Cultures] (CNCM held by the PASTEUR INSTITUTE, dated December 10, 1996, under No. I-1795.

The invention also relates to a host cell transformed by a recombinant plasmid as previously defined comprising the elements of regulation making possible the expression of the nucleotide sequence coding for the instant polypeptide in this host.

Such a cell is capable of expressing a SMBP according to the instant invention.

According to an advantageous embodiment, the host cell consists, in particular, in mammalian cell lines.

The invention also relates to antibodies directed specifically against the instant polypeptide, these antibodies being such that they recognize neither known α or β adrenergic, nor serotonine, nor dopamine receptors.

Advantageously, said new non-adrenergic receptor according to the invention constitute a tool for the selection of ligand participating in the activation or in the inhibition of these receptors.

The invention also relates to a method for assaying a substance for agonist or antagonist activity towards a polypeptide according to the invention, which method comprises:

- placing the substance in contact with tissue membrane proteins or a transformed host cell expressing a polypeptide according to the invention under conditions which permit binding between said polypeptide binding sites and an agonist or an antagonist thereto and

- measuring an appropriate transduction signal.

The invention also relates to a process for studying the binding affinity of a compound for a polypeptide according to the invention, which process comprises:

- transforming a host cell by an expression vector comprising a nucleotide sequence coding for the instant receptor,

- culturing said transformed host cell under conditions which permit the expression of said receptor encoded by said nucleotide sequence and the transfer of the expressed receptor polypeptide to the membrane of the said transformed host cell so that transmembrane sequences of said receptor polypeptide are embedded in the cell membranes of the transformed host cell;

- placing said transformed host cell in contact with said compound and

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- measuring the quantity of said compound bond to said receptor polypeptide.

The invention also relates to a process for studying the binding affinity of a compound for a polypeptide according to the invention, which process comprises:

- extracting membrane proteins corresponding to the instant receptor polypeptide from appropriate tissues or cells such as muscles,
- placing said membrane proteins in contact with said compound and
- measuring the quantity of said compound bond to said receptor polypeptide.

Functional roles of this polypeptide receptor would involve relaxation of depolarized-intestinal smooth muscle or inhibition of eosinophil chemotaxis.

Specific agonist for this new receptor will display at least a therapeutic potentiality in gastrointestinal diseases based on depolarized-tonus, allergic asthma and hypereosinophilic syndrome based on eosinophil accumulation.

Thus, the instant polypeptide receptor makes possible to develop drugs for at least gastrointestinal diseases based on depolarized-tonus, allergic asthma and hypereosinophilic syndrome.

Besides the foregoing arrangements, the invention also comprises other arrangements which will become apparent from the description which follows, reference being made to the attached drawings wherein:

. Figure 1: Preparative SDS-PAGE followed by autoradiography of 50 mg solubilized rat colon membranes photoaffinity-labeled with 0.5nM [^{125}I]-ICYP-diazirine in the presence of 10 µM 5-HT, 10 µM phentolamine and 20 µM propranolol;

. Figure 2: Analytical chemical cleavage of SMBP. The isolated-labeled protein of 34 kDa was incubated with distilled water (lane 1), 70% formic acid (lane 2), 10% cyanogen bromide in 70% formic acid (lane 3), 75% trifluoroacetic acid (lane 4) or 10% cyanogen bromide in 75% trifluoroacetic acid (lane 5) for 24 h at room temperature, separated by Tricine-SDS-PAGE followed by autoradiography. Arrows show 8, 10 and 12 kDa labeled fragments;

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. Figure 3: Preparative cyanogen bromide-cleavage of SMBP. The isolated-labeled proteins of 34 kDa were incubated with 10% cyanogen bromide in 70% formic acid for 24 h at room temperature. An aliquot of the cleaved-products was resolved on tricine-SDS-PAGE followed by autoradiography. Arrows show 8, 10 and 12 kDa labeled fragments.

. Figure 4: Analytical chemical cleavage of SMBP. Fig. 4a: the partially purified labeled proteins were incubated with distilled water (lane 1), 70% formic acid (lane 2) or 1% cyanogen bromide in 70 % formic acid (lane 3) for 24 h at room temperature or Fig. 4b: the partially purified labeled proteins were incubated with distilled water (lane 1), 70% formic acid (lane 2) for 72 h at 37°C, separated by Tricine-SDS-PAGE and subjected to autoradiography.

. Figure 5: Preparative acid-cleavage of SMBP. The isolated-labeled protein of 34 kDa was incubated with 70% formic acid for 72 h at 37°C. An aliquot of the cleaved-products was resolved on tricine-SDS-PAGE followed by autoradiography. Attows shows 8 kDa labeled fragment.

. Figure 6: Reverse-phase HPLC purification of the photoaffinity-labeled formic acid-cleaved 8 kDa fragment. The fragment isolated from tricine-SDS-PAGE gels was further purified by reverse-phase HPLC. Fragment was eluted from the C4 column with a linear gradient of 30-98% buffer B in 120 min (----). Radioactive profile for 8 kDa labeled fragment was shown (●). Based on the amount of recovered radioactivity, HPLC column recovery was 91.6%.

. Figure 7: Enzyme immunoassay (ELISA) of antiserum (●), preimmunized-serum (○) or affinity-purified antibody (■, α8-antibody) on plate coated with free peptide. Rabbit polyclonal antibody was raised against the synthetic peptide corresponding to the N-terminal sequence of the 8 kDa fragment.

. Figure 8: Immunoprecipitation of the solubilized photoaffinity-labeled SMBP. Solubilized-rat colon membranes photoaffinity-labeled with 1.5 nM [^{125}I]-ICYP-diazirine in the presence of 10 μM 5-HT, 10 μM phentolamine, 20 μM propranolol and

1.1 mM ascorbic acid were immunoprecipitated by 1/200 diluted-preimmunized serum (lane 1) or 10 µg of α8-antibody (lane 2).

. Figure 9: Western blotting of the rat colon membrane proteins. Lane 1 shows control (1/200 diluted-preimmunized serum was used). The 70 kDa band was detected by 2 µg/ml α8-antibody (lane 2). The detection was inhibited when antibody was preincubated with 10 µg/ml specific peptide (lane 3).

. Figure 10: Relationship between the efficacy of β-AR agonists in the rat colon and white adipocytes, in the presence of 10 µM phentolamine and 1 µM propranolol. The linear regression line of the four agonists, except SM-11044, is shown in figure ($r=0.97$, $p < 0.05$). The correlation coefficient, when calculated with SM-11044, was not significant ($r=0.87$, $p > 0.05$). Data represent mean pD2 values ± SEM (from Table 1).

. Figure 11: Time-course of association (○, solid line) and dissociation (●, dashed line) of 1 nM [^{125}I]-ICYP specific binding to rat colon membranes, in the presence of 10 µM 5-HT, 10 µM phentolamine and 20 µM propranolol. Reversibility of binding was obtained by the addition of 100 µM SM-11044 at equilibrium (30 min). Data represent mean of two experiments performed in duplicate.

. Figure 12: Total, non-specific and specific binding of [^{125}I]-ICYP to rat colon membranes, in the presence of 10 µM 5-HT, 10 µM phentolamine and 20 µM propranolol. Non-specific binding was determined in the presence of 100 µM SM-11044. Data represent mean of two experiments performed in duplicate. The inset shows Scatchard's plot of the specific binding ($r=-0.978$, $p < 0.001$). The Kd was 11.0 ± 0.95 nM and the Bmax was 716.7 ± 21.12 fmol/mg protein.

. Figure 13: Displacement of 1 nM [^{125}I]-ICYP specific binding to rat colon membranes by (a) catecholamines, 5-HT and (b) stereo-isomers of SM-11044, in the presence of 10 µM 5-HT, 10 µM phentolamine and 20 µM propranolol. Data represent mean of two to four experiments performed in duplicate.

. Figure 14: SDS-PAGE followed by autoradiography of solubilized rat colon membranes photoaffinity-labeled with 1.5 nM [^{125}I]-ICYP-diazirine in the presence

of 10 µM 5-HT, 10 µM phentolamine and different competitors. Lane 1, control; lane 2, displacement by 20 µM propranolol; lane 3, displacement by 20 µM propranolol and 100 µM BRL-37344; lane 4, displacement by 20 µM propranolol and 100 µM SM-11044.

. Figure 15: Two-dimensional SDS-PAGE followed by autoradiography of solubilized rat colon membranes photoaffinity-labeled with 1.5nM [^{125}I] ICYP-diazirine in the presence of 10 µM 5-HT, 10 µM phentolamine and 20µM propranolol.

. Figure 16: Tryptic cleavage of the photoaffinity-labeled rat colon membranes. The partially purified labeled proteins were incubated without (lane 1) or with 50 µg trypsin (lane 2) for 24 h at 37°C, separated by Tricine-SDS-PAGE and subjected to autoradiography.

. Figure 17: Displacement of 1 nM [^{125}I]-ICYP specific binding to rat skeletal muscle membranes by SM-11044 and (-)isoproterenol, in the presence of 10 µM 5-HT, 10 µM phentolamine and 20 µM propranolol. Data represent mean ± S.E.M of two experiments performed in duplicate.

. Figure 18: Human multiple tissue northern blot hybridized with labeled 300 bp probe. Washes at 2 x SSC, 0.05% S.D.S, at room temperature and exposure on Hyperfilm MP with two intensifying screens at -80°C for three days. (A) Northern blot hybridization was performed on polyadenylated mRNA from 8 different smooth and striated human muscles. (B) similar analysis with a variety of non muscular tissues (heart, brain, placenta, lung, liver, kidney and pancreas). On the left: scale indicates RNA molecular weight marker in kilobases (Kb).

. Figures 19, 20 and 21 illustrate the restriction map of SEQ ID NO:14 (all sites: figure 19; unique sites only: figure 20 and figure 21).

. Figure 22 illustrates a sequence comparison with known proteins (Arabidobsis protein, hMP70 protein, p76 protein, D87444 protein and Emp70 protein).

. Figure 23 illustrates (A) a comparison of hydropathy profiles (Kyte & Doolittle) by GeneJockey Sequence Processor programm between SMBP and the homologous proteins D87444, Hu p76, hMP70 and Emp70 from yeast and Arabidobsis protein. (B) Comparison of the hydropathy profiles (Kyte & Doolittle method) of the C-

terminal hydrophobic region between SMBP and the homologous proteins D87444, Hu p76 and Emp70 of yeast.

. Figure 24 illustrates the sequences corresponding to the hydrophobic stretches (boxes).

. Figure 25 illustrates immunoprecipitation of (^{125}I)-iodinated cell membrane proteins by α8-antibody: lane 1: COS cells transfected with a vector containing the angiotensin receptor AT2R; lane 2: COS cells transfected with a vector containing the SMBP nucleotide sequence.

Example 1: Isolation and characterization of the instant receptor in rat colon smooth muscle membranes.

1) **Materials and Methods**

SM-11044 ((L)-threo-3-(3,4-dihydroxyphenyl)-N-[3-(4-fluorophenyl) propyl] serine pyrrolidine amide hydrobromide) and (±)-cyanopindolol were synthesized at Sumitomo Pharmaceuticals (Osaka, Japan). (-)-3-[^{125}I] iodocyanopindolol ([^{125}I]-ICYP) and (±)-3 [^{125}I]-iodocyanopindolol-diazirine ([^{125}I]-ICYP-diazirine) were purchased from Amersham (Buckinghamshire, England). All other materials were reagent grade.

Rat colon membrane preparation

Frozen rat colons (SD strains, male and female) were purchased from Pel-Freeze Biologicals (Arkansas, USA). Membranes from colon smooth muscles were prepared as essentially described by Ek et al., 1986, with the slight following modifications. The colon segment was washed in ice-cold Tris-saline (10 mM Tris/HCl, 154 mM NaCl, (pH 7.4)), cut open longitudinally and the mucosa was removed by scrubbing with a glass slide on ice-cold plastic plate. The smooth muscle preparations were homogenized with a Polytron homogenizer for 1 min. The homogenate was filtered through a gauze and centrifuged (1,500 x g for 20 min at 4°C), the supernatant was collected and centrifuged (50,000 x g for 20 min at 4°C). The pellet comprising the membranes was resuspended in Tris-saline and was stored at -80°C until use. The protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad USA).

Binding assays in rat colon membranes

Saturation binding studies were performed in a final volume of 200 µl of Tris-saline containing 50 µg membrane proteins and different concentrations (0.05-25 nM) of [^{125}I]-ICYP, in the presence of 10 µM serotonine (5-HT), 10 µM phentolamine, 20 µM (±) propranolol and 1.1 mM ascorbic acid (pH 7.4), to block possible 5-HT receptors, ARs and oxidation of catecholamines, respectively. The [^{125}I]-ICYP was used after removing methyl alcohol by compressed air to avoid the influence of the solvent. Incubations were carried out at 37°C for 30 min in a shaking water-bath incubator and terminated by addition of 4 ml of ice-cold Tris-saline followed by rapid filtration under vacuum on Whatman GF/B filter presoaked in Tris-saline containing 0.1% polyethyleneimine (pH 7.4). The filters were washed three times with 4 ml of ice-cold Tris-saline, transferred to plastic tubes and counted in a γ-counter.

Photoaffinity labeling of the rat colon membranes

Photoaffinity labeling was performed in a final volume of 10 ml of Tris-saline containing 50 mg membranes, 0.5 nM [^{125}I]-ICYP-diazirine, supplemented with 10 µM 5-HT, 10 µM phentolamine, 20 µM (±) propranolol and 1.1 mM ascorbic acid (pH 7.4) were incubated at 37°C for 60 min in the dark in a shaking water-bath incubator; the reaction was terminated by addition of 20 ml of ice-cold Tris-saline followed by a rapid centrifugation (50,000 x g for 10 min at 4°C). The membranes were resuspended in 2-3 ml of the same buffer and irradiated with a UV lamp for 10 min with cooling by circulating water (Guillaume et al., 1994). The labeled membranes were diluted with 20 ml of ice-cold Tris-saline, centrifuged (50000 x g for 30 min at 4°C). The labeled membranes were immediately denatured in SDS-reducing buffer (5% SDS, 1% 2β-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue, 50 mM Tris/HCl, pH 6.8) for 1 h or more at room temperature before electrophoresis.

Preparative SDS-PAGE and extraction of the photoaffinity-labelled proteins

Preparative SDS-PAGE was performed with a large size (160 mm width x 200 mm height x 3 mm thickness) of 12% separating and 4% stacking polyacrylamide

gels (40% T, 2.6% C) under reducing conditions essentially according to the methods of Laemmli, 1970. After electrophoresis, the gels were packed in a plastic bag and auto-radiographed for 3 days at 4°C on X-OMAT™ AR film (Eastman Kodak Co., USA). The photoaffinity labeled proteins were extracted by passive extraction, as follows. The radio-active 34 kDa band was cut out and crushed to small pieces of less than 3x3x3 mm^3 by squeezing out using 10 ml disposable plastic syringe (Terumo, Japan). The gels were immersed in twice volume of 100 mM Tris/HCl (pH 8.0) containing 0.1% SDS (extraction buffer), and incubated for 16 h at 37°C with rotating. The extract was recovered using a SPIN-XII (0.45 µm pore size, Costar, USA) at 1,500 x g for 30 min. The remaining gel pieces were again immersed in twice volume of extraction buffer, incubated for 2 h at 37°C with rotating, and the extract was recovered as described above. The two extracts were combined and concentrated to at maximum 0.5 ml using Centriprep 10 and Centricon 10 (Amicon, USA) and kept at -20°C.

Chemical cleavage of the extracts from preparative SDS-PAGE and purification by HPLC

The 34 kDa photoaffinity-labeled protein extracted from the preparative SDS-PAGE were washed twice by distilled water using Centricon 10 and lyophilized by vacuum concentrator and treated with 200 µl of 70% formic acid or 10% CNBr/70% formic acid for 24 h at room temperature, or 70% formic acid for 72 h at 37°C in the dark. The cleaved products were diluted with 500 µl distilled water and lyophilized. This washing procedure was repeated three times. The cleaved products were dissolved in SDS-reducing buffer and neutralized by addition of aliquots of 30% NaOH until changing the coloration to blue, and were separated by tricine-SDS-PAGE. The gels were dried and autoradiographed. The labeled bands were cut out, passively extracted and blotted on PVDF membranes by centrifugation (ProSpin™, Applied Biosystems, USA). The membranes were washed 3 times with 1 ml of 20% methanol to remove SDS and salts. The fragments were extracted by 200 µl of 75% hexafluoro-isopropanol. Each elution was dried to 20 µl in vacuum concentrator, dissolved in 75µl DMSO and 75 µl of starting buffer (15% acetonitrile-15% isopropanol-0.5% TFA; buffer A) and loaded on a C4

reverse phase column (Aquapore Butyl BU-300, 2.1 mm ID, 10 mm length, Applied Biosystems). Separation was carried out by a 120 min gradient elution at 40°C with 50% acetonitrile-50% isopropanol containing 0.5% TFA (buffer B) at a flow rate of 0.35ml/min using a Waters 625 LC System. The gradient started from 30% to 98% buffer B. The elution of fragments was monitored by the absorbance at 210 and 275nm, and the elution of radioiodinated products was monitored by γ-counting of the fractions.

Tricine-SDS-PAGE

Chemical cleaved fragments were separated on a Tricine gel system under reducing conditions (Schägger H. et al., 1987) using 18% polyacrylamide separating gel containing 10.7% glycerol. The gels aged for 16 h to allow for decomposition of reactive chemical intermediates after polymerization.

Amino acid sequencing

Amino acid sequence determination was performed by Edman degradation, 1967, with an Applied Biosystems 473A protein sequencer. Samples were applied to precycled filters, coated with Polybrene (Biobrene, Applied Biosystems) to reduce peptide-wash-out and to improve initial yields.

Antibody preparation

Antibody was prepared as essentially described by Guillaume et al. (Eur. J. Biochem., 1994, **224**, 761-770).

Briefly, based on the determined amino acid sequences, peptides were synthesized adding a cysteine residue at C-terminal residue to facilitate coupling to the carrier protein (Keyhole limpet hemocyanin, KLH). The synthetic peptides were conjugated to KLH through their cysteine residues. A 0.4 mg of the peptide-conjugate, suspended in Freund's complete adjuvant, was intradermally injected into rabbit. Boosters were given 4 times at 2 weeks intervals by injection of a 0.2 mg of the peptide-conjugate suspended in Freund's incomplete adjuvant. Two weeks later the final immunization, antiserum was recovered from whole blood.

Antibody was purified by affinity chromatography on a column containing the synthetic peptide coupled to activated thiol-Sepharose-4B (Pharmacia)

through a cysteine at C-terminal residue, and the antibody titer level against the free peptide without conjugation to KLH was determined by ELISA.

Immunoprecipitation

Total amounts of 10 mg membranes were photoaffinity-labeled with 1.5 nM [^{125}I]-ICYP-diazirine in the presence of 10 µM, 5-HT, 10 µM phentolamine, 20 µM propranolol and 1.1 mM ascorbic acid in 10 ml of Tris-saline (pH 7.4). Membranes were solubilized at 1 mg membrane protein/ml of Tris-saline containing 2% n-octylglucoside (n-octyl β-D-glucopyranoside, Sigma) for 2 h on ice with occasional mixing. The solubilized-proteins were separated from the insoluble material by centrifugation (200,000 x g, 30 min at 4°C). The proteins were treated with 8 M urea for 1 h at room temperature with occasional mixing and were washed 5 times with Tris-saline using Centricon 10. The solubilized-membrane proteins were dissolved in 1ml Tris-saline containing 0.1% Tween-20 and were incubated with 10 µg antibody and 50 µl protein-A-agarose beads (Boehringer-Mannheim, Germany) for 16 h at 4°C with rotating. The precipitant was gently washed 5 times with ice-cold Tris-saline containing 0.1% Tween-20 and denatured in SDS-reducing buffer for more than 1 h at room temperature. The immunoprecipitated proteins were subjected to 12% SDS-PAGE and autoradiographed.

Western blotting

Photoaffinity-labeled membranes (40 µg protein) were separated by 12% SDS-PAGE. Electrotransfer of proteins onto nitrocellulose was carried out essentially according to Towbin et al., 1979, on a Trans-Blot SD apparatus (Bio-Rad) for 1 h at a current intensity of 1mA/cm^2. Nitrocellulose membranes were washed three times with Dulbecco's phosphate buffered saline (PBS) containing 0.2% Tween-20 and were saturated in PBS containing 5% skimmed milk powder and 0.2% Tween-20 for 1 h at room temperature. Antibody (2 µg/ml in PBS containing 1% skimmed milk powder and 0.2% Tween-20; buffer C) was allowed to react for 16 h at 4°C.

After three times washing in buffer C, the nitrocellulose strips were incubated for 45 min at room temperature with peroxidase-conjugated affinity-purified Goat anti-rabbit IgG (Jackson Immuno-Research Laboratories, USA) at a 1/2500 dilution in

buffer C, washed three times in buffer C. After washing in PBS containing 0.2% Tween-20, reactive bands were visualized with an ECL kit (Amersham, England). In inhibition experiments, antibody was preincubated for 2 h at 37°C with free peptide at a concentration of 10 µg/ml in buffer C.

2) **Results**

Extraction of the photoaffinity-labeled SMBP

Membrane proteins of 2.0 g were collected from 600 rat colon smooth muscles. The ligand binding activity of SMBP was assessed by [^{125}I]-ICYP under blockade of adrenergic and serotonine receptors. Scatchard plot analysis revealed a single class of binding sites with a dissociation constant (Kd) of 7.22 ± 0.007nM and a maximum number of binding sites (Bmax) of 1.13 ± 0.071 pmol/mg membrane protein (two independent experiments performed duplicate, expressed as means ± SD).

The SMBP was too hydrophobic to separate by any column chromatography such as reverse-phase HPLC with C4 column (Aquapore Butyl BU-300, Applied Biosystems), ion exchange chromatography (Aquapore Weak Anion AX-300, Applied Biosystems), chromatofocusing (PBE 94 and Polybuffer 74, Pharmacia), hydroxyapatite chromatography (BioGel HPHT, Bio-Rad). Preparative SDS-PAGE was performed to separate SMBP just after the photoaffinity labeling. Fifty mg of the labeled-membranes could be loaded on a set of polyacrylamide gels without serious diffusion of the 34 kDa labeled-SMBP (figure 1). The passive extraction of 34kDa bands yielded 79.3-86.2% of the total radioactive proteins in gels.

Chemical cleavage, purification and sequencing

Chemical cleavage has some advantage in contrast to proteolytic digestion; it avoids contamination by protease itself, and produces limited numbers of large fragments. Analytically, each 1 mg of the labeled 34 kDa protein was treated with 10% CNBr in 70% formic acid or in 75% TFA to compare the effect of acid. In formic acid condition, CNBr generated three labeled fragments of 8, 10 and 12 kDa, and formic acid alone generated a single 8 kDa labeled fragment. In the acid condition with TFA, most of

the labeling was dissociated by acid itself, a single 10 kDa labeled fragment was observed by CNBr cleavage (figure 2).

The extract of the labeled 34 kDa protein from 400 mg membranes (411794 cpm) was preparatively cleaved by CNBr/formic acid, and an aliquot of the cleaved-products was resolved on tricine-SDS-PAGE gels. Three labeled fragments of major 12 kDa and minor 8 and 10 kDa were observed on autoradiogram of coomasie blue stained gels (figure 3).

Cleavage at methionine residues by CNBr/formic acid treatment for 24 h at room temperature of the photoaffinity-labeled 34 kDa protein yielded three labelled-fragments (8, 10 and 12 kDa, Fig. 4a, lane 3). Treatment by formic acid alone generated a single 8 kDa fragment (Fig. 4a, lane 2), and the density of the 8 kDa band increased upon prolonged incubation (for 72 h at 37°C, Fig. 4b, lane 2).

The extract of the labeled 34 kDa protein from 400 mg membrane (381198 cpm) was preparatively cleaved by formic acid, and an aliquot of the cleaved-products was resolved on tricine SDS-PAGE gels. A single labeled-fragment of 8kDa was observed on autoradiogram of coomasie blue stained-gels (figure 5). The radioactive 8 kDa fragment (total 21400 cpm) in preparative scale was extracted by passive extraction from tricine-SDS-PAGE gels without coomasie blue staining, and was blotted on PVDF membranes (19581 cpm). The fragment was extracted from PVDF membranes (10045 cpm) and further purified by reverse-phase HPLC. One radioactive peak was observed at 62% buffer B (fraction n° 27 and 28; total 3239 cpm, figure 6). Total recovery yield of the initial radioactivity was 91.6%. The peak fractions were submitted to protein sequencer, and the resulting amino acid sequence was determined as follows:

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1     5     10    15
|     |     |     |
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(D) P X F F Q H R I H V F S I F N H (SEQ ID NO:6)

Parenthesis; expected amino acid

X; undetermined amino acid.

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Analytical CNBr-cleavage indicated that cleavage at methionine residue in the presence of TFA, which improve the cleave at CNBr-resistant bonds such as Met-Thr or Met-Ser (Fontana A. et al., 1986), generated a single 10 kDa fragment.

In formic acid condition, CNBr generated three labeled fragments of 8, 10 and 12 kDa, and formic acid alone generated a single 8 kDa labeled fragment. These data suggest that 12 kDa fragment contains a CNBr-resistant methionine residue cleaved to 10 kDa by CNBr/TFA and that the 8 kDa fragment by formic acid alone is a product by cleavage at acid-sensitive bond such as Asp-Pro.

Immunoprecipitation and Western blotting

The peptide corresponding to the N-terminal sequence of the 8 kDa fragment (Acetyl-FFQHRIHVFSIFNHC) was coupled to KLH and the conjugate was used to raise antibody with high titer. The antibody response was observed at $2x10^{-5}$ dilution of antiserum and at 0.08 µg/ml of affinity purified antibody (α8-antibody) as assessed by ELISA against free peptide without conjugation to KLH (figure 7).

The synthetic peptide corresponding to the 8 kDa-fragment was hydrophobic and could not be dissolved in a buffer without dimethyl sulfoxide. Initially, the labeled 34 kDa protein, extracted from preparative SDS-PAGE gels, was used after removing SDS, but no labeled protein was immunoprecipitated. After solubilization of the photoaffinity-labeled membranes by n-octylglucoside followed by denaturation with urea, the α8-antibody immunoprecipitated the labeled 34 kDa SMBP (figure 8).

The α8-antibody recognized only a 70 kDa band by western blotting. The specificity of the antibody was demonstrated by the ability of the free peptide to inhibit the binding of the antibody to the 70 kDa protein (figure 9). In a separate experiment, photoaffinity-labeled SMBP was purified by two-dimensional electrophoresis in preparative scale, and the 34 kDa labeled spot in gels was isolated, extracted and subjected to SDS-PAGE. Two labeled bands of 34 and 70 kDa derived from 34 kDa were observed, indicating that the 70 kDa protein could be dimer.

Example 2: Pharmacological properties of the rat receptor according to example 1.

Catecholamine-induced relaxant responses which are resistant to blockade of α-, β1- and β2-adrenoceptors (ARs) have been described in a number of gastro-intestinal smooth muscle preparations, such as guinea pig ileum (Bond R.A. et al., 1988), rat proximal colon (Croci T. et al., 1988), rat distal colon (McLaughlin D.P. et al., 1990), rat gastric fundus (McLaughlin D.P. et al., 1991) and rat jejunum (Van der Vliet A. et al., 1990). Manara et al., 1990, actually reported that the phenylethanolamino-tetralines-stimulated rat colon relaxation paralleled rat adipocyte lipolysis, suggesting that this response predominantly involved the β3-AR.

1) **Materials and Methods**

Chemicals

SM-11044 ((L)-threo-3-(3,4-dihydroxyphenyl)-N-[3-(4-fluorophenyl) propyl] serine pyrrolidine amide hydrobromide), SM-14786 ((D)-threo isomer of SM-11044), SM-14011 ((DL)-threo-isomer of SM-11044), SM-14010 ((DL)-erythro-isomer of SM-11044), BRL-35135A ((R*R*)-(+)-4-[2'-[2-hydroxy 2-(3-chlorophenyl) ethyl amino] propyl] phenoxyacetic acid methyl ester), BRL-37344 (acid metabolite of BRL-35135A) and ((^{125}I)-cyanopindolol were synthesised at Sumitomo Pharmaceuticals (Osaka, Japan). CGP-12177A and CGP-20712A were gifts from Ciba-Geigy Corporation (Basal, Switzerland). ICI-198157 ((RS)-4-[2-[(2-hydroxy-3-phenoxypropyl) amino] ethoxy] phenoxyacetic acid methyl ester), ICI-201651 (acid metabolite of ICI-198157) and ICI-215001 ((S)-isomer of ICI-201651) and ICI-118551 were obtained from Zeneca Pharmaceuticals (Macclesfield, England). SR-58611A ((RS)-N-(7 carbethoxymethoxyl-1,2,3,4-tetrahydronaphth-2-yl)-2-hydroxy-2-(3 chlorophenyl) ethanamine hydro-chloride) was a gift from Sanofi-Midy (Milano, Italy). (+)-Carazolol was obtained from Boehringer Mannheim (Mannheim, Germany). (+)-Bupranolol was a gift from Schwarz Pharma (Monheim, Germany). (-)-3-[^{125}I] iodocyanopindolol ([^{125}I]-ICYP) and (+)-3 [^{125}I]-iodocyanopindolol-diazirine ([^{125}I]-ICYP-diazirine) were purchased from Amersham (Buckinghamshire, England). All other drugs were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Relaxation of rat colon

Rat colon segment (2 cm) was suspended in organ bath containing 10 ml modified-Tyrode solution (Sugasawa T. et al., Eur. J. Pharmacol., 1992, 216, 207-215). The Tyrode solution contained 0.5 μM atropine, 0.5 μM desmethylimipramine, 30 μM hydrocortisone, 30 μM ascorbic acid, 10 μM phentolamine and 1 μM propranolol throughout study, in order to inhibit spontaneous contraction, neuronal and extra-neuronal uptake of norepinephrine, oxidation of catecholamines, possible α, β1- and β2-AR effects, respectively.

The relaxant action of agonists was determined by measuring relaxation of KCl (100 mM)-induced tonus evoked by cumulative addition of the agonists as described previously (Sugasawa T. et al. cited above). In the case of testing the effect of cyanopindolol, it was added 5 - 10 min before the addition of agonist.

Lipolysis in rat white adipocytes

White adipocytes were isolated from epididymal fat pads of male Wistar rats (190 - 230 g) and lipolysis was determined according to the previous report (Sugasawa T. et al. cited above). The cells were preincubated for 5 min at 37°C in the presence of 30 μM ascorbic acid, 10 μM phentolamine and 1 μM propranolol.

Agonists were then applied and incubated for 90 min. In the case of testing the effect of cyanopindolol, it was added 5 min before the addition of agonist.

Schild plot

Agonist concentration-ratios (CR) were determined from the EC_{50} values of the concentration-response curves of agonists with or without cyanopindolol, according to the method of Arunlakshana et al., 1959.

Linear regression analysis was used to estimate the pA_2 value and slope of the line, after confirming that the regression was linear and the slope was not significantly different from unity (Cochran-cox test, $p > 0.05$). The EC_{50} values were calculated using the computer program, InPlot™.

Statistical analysis

Results are expressed as mean ± SEM. Statistical significance between two data sets was examined by Student's t-test or Cochran-cox test, depending on the homogeneity of the variances. Duncan's multiple range test was used for multiple data sets. A probability level of $p < 0.05$ was considered to be significant.

Membrane preparation

Membranes from the colon smooth muscle and from skeletal muscle were prepared from male Wistar rats (300 - 360 g) as essentially mentioned in example 1.

Binding assays in membranes

Saturation binding studies were performed in a final volume of 200 µl of Tris-saline containing 50 µg membrane proteins and different concentrations (0.05-25 nM) of [^{125}I]-ICYP, supplemented with 10 µM serotonine (5-HT), 10 µM phentolamine, 20 µM propranolol and 1.1 mM ascorbic acid (pH 7.4), to block possible 5-HT receptors, ARs and oxidation of catecholamines, respectively. The [^{125}I]-ICYP was used after removing methyl alcohol by compressed air to avoid the influence of the solvent. Incubations were carried out at 37°C for 30 min in a shaking water-bath incubator and terminated by addition of 4 ml of ice-cold Tris-saline followed by rapid filtration under vacuum on Whatman GF/B filter presoaked in Tris-saline containing 0.1% polyethyleneimine (pH 7.4). The filters were washed three times with 4 ml of ice-cold Tris-saline, transferred to plastic tubes and counted in a γ-counter.

Competition assays were performed against 1 nM [^{125}I]-ICYP. Non-specific binding was determined in the presence of 100 µM SM-11044. The inhibition constant, Ki, of a ligand was calculated using the equation described by Cheng and Prusoff (Biochem. Pharmacol., 1973, **22**, 3099-3108). Hill coefficient was calculated by linear regression using saturation experiment data. Pseudo-Hill coefficient and IC_{50} were determined by the computer program, InPlot™ (GraphPad Software, CA, USA).

Photoaffinity labeling of the membranes

Photoaffinity labeling was performed in a final volume of 1 ml of Tris-saline containing 0.5 mg membranes, 1.5 nM [^{125}I]-ICYP-diazirine, supplemented with

10 µM 5-HT, 10 µM phentolamine, 20 µM propranolol and 1.1 mM ascorbic acid (pH 7.4). Incubations were carried out in the presence or absence of competitor at 37°C for 45 min in the dark in a shaking water-bath incubator and terminated by addition of 10 ml of ice-cold Tris-saline followed by a rapid centrifugation (150,000 x g for 10 min at 4°C). The membranes were irradiated with a UV lamp for 5 min with cooling by circulating water. The labeled membranes were diluted with 10 ml of ice-cold Tris-saline, centrifuged (150,000 x g for 30 min at 4°C), and the pellet was resuspended in Tris-saline and kept at -80°C.

SDS-PAGE

SDS-PAGE was performed under reducing conditions essentially as described by Laemmli, 1970, using 12% polyacrylamide gels (40% T, 2.6% C). The photoaffinity-labelled membranes were incubated in SDS-sample buffer (5% SDS, 1% 2β-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue, 50 mM Tris/HCl, (pH 6.8)) for at least 1 h at room temperature. After electrophoresis, the gels were dried and autoradiographed on X-OMAT™ AR film (Eastman Kodak Co., NY, USA), as specified in example 1, chapter «preparative SDS-PAGE».

Two-dimensional PAGE of photoaffinity-labeled membranes

Photoaffinity-labeled membranes in the presence of 10 µM 5-HT, 10µM phentolamine and 20 µM propranolol were solubilized in IEF-sample buffer (8 M urea, 0.3% SDS, 5.6% Triton X-100, 2.8% 2β-mercaptethanol, 1.1% Bio-Lyte 5/8 ampholyte and 0.6% Bio-Lyte 8/10 ampholyte (Bio-Rad)) and 30 µg of membrane proteins were submitted to IEF electrophoresis in a 5 - 10 pI range of 4% polyacrylamide tube gels containing 2.0% Bio-Lyte 5/8 ampholyte, 1.0% Bio-Lyte 8/10 ampholyte, 8 M urea and 2% Triton X-100. The second dimension was conducted on SDS-PAGE of 9% polyacrylamide gels. The gels were then dried and submitted to autoradiography as described above.

Cleavage by endoglycosidase or N-glycopeptidase F

Photoaffinity-labeled membranes in the presence of 10 µM 5-HT, 10µM phentolamine and 20 µM propranolol were treated with N glycopeptidase F (PNGase F,

EC 3.2.2.18) or endoglycosidase (Endo Hf, EC 3.2.1.96), using kits according to the manufacturer's specifications (New England Bio-Labs, MA, USA). Briefly, the membranes were solubilized in 0.5% SDS and 1% 2β-mercaptethanol, and 40 µg of membrane proteins were incubated with 5000 units of PNGase F in the presence of 1% NP-40 or with 2000 units of Endo Hf for 3 h at 37°C. The digested samples were subjected to SDS-PAGE of 12% polyacrylamide gels. The gels were then dried and submitted to auto-radiography as described above.

Wheat germ agglutinine (WGA) - sepharose chromatography

Photoaffinity-labeled membranes in the presence of 10 µM 5-HT, 10µM phentolamine and 20 µM propranolol were solubilized in 1% Triton X 100 in Tris-saline at 4°C for 16 h. The solubilized material was collected after centrifugation (200,000 x g for 1 h at 4°C) and diluted to 0.1% Triton X-100 by Tris-saline. One milliliter gel bed volume of WGA-sepharose 6MB (Sigma) was washed and equilibrated with 30 ml of 0.1% Triton X-100 in Tris-saline (buffer A), and 1 ml of solubilized material containing 200 µg membrane proteins was loaded at room temperature. The unretained fraction was recycled three times. After washing with 10 ml of buffer A, the bound material was eluted with 5 ml of 300 mM N-acetyl-D-glucosamine (Merck) in buffer A. The fractions were subjected to SDS-PAGE of 12% polyacrylamide gels. The gels were then dried and submitted to autoradiography as described above.

Tryptic cleavage

The photoaffinity-labeled membranes were subjected to SDS-PAGE of 12% polyacrylamide gels. The gels were then dried and submitted to autoradiography as described above. The radioactive band at 34 kDa was excised, immersed in distilled water and minced to small pieces (2 mm width x 2 mm height). The isolated gel pieces corresponding to 800 µg membrane proteins was digested in 500 µl of 100 mM Tris/HCl (pH 8.0) containing 0.1% SDS and 50 µg trypsin (EC 3.4.21.4, Type IX from Porcine Pancreas, Sigma) for 24 h at 37°C according to the method of Kawasaki H. et al., 1990. After digestion, the supernatant was recovered and filtrated using a SPIN-X filter (0.45 mm pore size, Costar, MA, USA). The gel pieces were crushed through a nylon mesh (200

mesh) by centrifugation for 10 min at 14,000 x g. A 2-fold volume of 100 mM Tris/HCl containing 0.1% SDS was added to the crushed gels, and a second extraction was performed by incubation for 2 h at 37°C with rotating. After incubation, the supernatants were recovered by SPIN-X filter. The two extracts were combined, vacuum concentrated and submitted to Tricine-SDS-PAGE.

Chemical cleavage

The 34 kDa photoaffinity-labeled protein was isolated by SDS-PAGE and extracted with 100 mM Tris/HCl (pH 8.0) containing 0.1% SDS as described above. The extracts were combined and concentrated by Centricon 10 (Amicon, MA, USA) and washed twice by distilled water. The extracts were lyophilised by vacuum concentrator and treated with 200 µl of 70% formic acid or 1% CNBr/70% formic acid for 24 h at room temperature, or 70% formic acid for 72 h at 37°C in the dark. The cleaved products were diluted with 500 µl distilled water and lyophilised. This washing procedure was repeated three times. The cleaved products were separated by Tricine SDS-PAGE.

Tricine-SDS-PAGE

Tryptic and chemical cleaved fragments were separated on a Tricine gel system under reducing conditions (Schägger H. et al., 1987) using 18% polyacrylamide separating gel containing 10.7% glycerol. After electrophoresis, the gels were stained with 0.25% Coomassie brilliant blue R-250 (Sigma) in 40% methanol and 10% acetic acid, and destained in 10% acetic acid. The gels were then dried and submitted to autoradiography as described above.

2) **Results**

Functional studies in rat colon and white adipocytes

Under blockade of α-, β1- and β2-ARs (in the presence of 10 µM phentolamine and 1 µM propranolol), a number of β–AR agonists relaxed KCl-induced tonus in rat colon smooth muscle segment, giving a rank order of potency of BRL-37344 > SM-11044 >> isoproterenol >> norepinephrine = epinephrine (Table 1).

TABLE 1

Agonist efficiency in rat colon relaxation and rat white adipocyte lipolysis in the presence of 10 μM phentolamine and 1 μM propranolol

Agonist	Rat colon			Rat white adipocytes		
	pD_2	IA	n	pD_2	IA	n
(-)-isoproterenol	6.64±0.22	1.00±0.063	5	5.86±0.07	1.00±0.037	5
(-)-norepinephrine	5.85±0.27	0.85±0.168	4	5.40±0.10	1.02±0.058	5
(-)-epinephrine	5.92±0.06	0.86±0.137	6	5.16±0.06	0.91±0.036	5
BRL-37344	7.50±0.18	1.00±0.126	8	7.25±0.09	0.72±0.0333**	5
SM-11044	7.29±0.21	1.48±0.166*	7	5.96±0.11	0.86±0.054	5

Statistical significance between IA values; * $p < 0.05$, ** $p < 0.01$ vs isoproterenol (Duncan's multiple range test).

The IA value of SM-11044 was significantly higher than that of isoproterenol (Duncan's multiple range test, $p < 0.05$), indicating different modes of action. In rat white adipocytes, the same agonists stimulated lipolysis with a rank order of potency of BRL-37344 >> SM-11044 = isoproterenol > norepinephrine > epinephrine (Table 1). The linear regression line for isoproterenol, norepinephrine, epinephrine and BRL-37344 reveals a significant correlation ($r=0.97$, $p < 0.05$) between agonist induced rat colon relaxation and adipocyte lipolysis (Fig. 10), suggesting that both effects predominantly involve the same atypical β-, that is β3-AR stimulation. In contrast to the four ligands, SM-11044 stimulated colon relaxation more efficiently than adipocyte lipolysis (Fig. 10). Indeed, the correlation coefficient ceased to be significant when linear regression was analyzed with all agonists including SM-11044 ($r= 0.87$, $p > 0.05$). These data suggest that SM-11044 acts on β3-AR and additional functional site that mediates relaxation in rat colon. Antagonism of cyanopindolol for SM-11044 and for isoproterenol was compared in both preparations. Cyanopindolol itself, up to the concentration of 10 μM used here, had no effect on the degree of tonus induced by KCl in rat colon and did not stimulate lipolysis in rat white adipocytes. Cyanopindolol antagonised agonist-induced rat colon relaxation in a concentration-dependent manner, with pA_2 values for SM-11044 of 8.31 (slope = 0.78) and for isoproterenol of 7.65 (slope = 1.03) (Table 2).

TABLE 2

pA_2 values for cyanopindolol in rat colon and rat white adipocytes in the presence of 10 μM phentolamine and 1 μM propranolol.

Agonist	Rat colon			Rat white adipocytes		
	pA_2	Slope	n	pA_2	Slope	n
(-)-isoproterenol	7.65±0.48	1.03±0.08	5	7.44±0.61	1.08±0.10	4
SM-11044	8.31±0.88	0.78±0.11	5	7.32±1.51	0.96±0.21	4

Cyanopindolol also antagonized agonist-induced rat white adipocyte lipolysis in a concentration-dependent manner, with pA_2 values for SM 11044 of 7.32 (slope = 0.96) and for isoproterenol of 7.44 (slope = 1.08) (Table 2). The similar pA_2 values for isoproterenol in colon (7.65), SM-11044 in adipocytes (7.32) and isoproterenol in adipocytes (7.44) with the slopes close to unity, indicating the competitive antagonism of cyanopindolol for both agonists binding to β3-AR. All slopes of Schild plots were not significantly different from unity. However, only the slope for SM-11044 in rat colon (0.78) seemed to be lower than unity with high pA2 value (8.31), suggesting that SM-11044 and cyanopindolol compete not only binding to β3-AR but also to additional functional site on rat colon.

Binding assays in rat colon membranes

In order to identify the predicted functional site, being competed by SM-11044 and cyanopindolol, binding studies in rat colon smooth muscle membranes were performed using [^{125}I]-ICYP for radioligand and SM-11044 for non-specific binding determination, under blockade of serotonine, α-, β1-, β2- and also β3-adrenergic receptors (in the presence of 10 μM 5-HT, 10 μM phentolamine and 20 μM propranolol). The time course of specific binding of [^{125}I]-ICYP (1 nM) to rat colon membranes was illustrated in Fig. 11. Specific binding achieved equilibrium levels at 30 min (82.7 ± 1.9%, n=2), and was reversed by addition of SM-11044. The results of a saturation experiment with increasing amount of [^{125}I]-ICYP, carried out at equilibrium (30 min incubation), are illustrated in Fig. 12. Scatchard plot analysis revealed a single class of binding sites with a

dissociation constant (Kd) of 11.0 ± 0.95 nM, and a maximum number of binding sites (Bmax) of 716.7 ± 21.12 fmol/mg protein ($r=-0.978$, $p < 0.001$). Hill plot analysis of the saturation curve yielded a coefficient of 0.99 ± 0.03 ($r=0.998$, $p < 0.0001$), indicating the absence of cooperativity.

In competition binding studies, specific binding was not displaced by isoproterenol, norepinephrine, epinephrine, dopamine nor 5-HT, up to the concentration of 1 mM (Fig. 13a, Table 1). The competition binding by isomers of SM-11044 was stereo-selective, SM-14011 (the racemic threo isomer, Ki 2.0 μM) being 15 times more effective than SM-14010 (the racemic erythro-isomer, Ki 29.3 μM) (Fig. 13b, Table 3). The β1-AR antagonist, CGP20712A and the β3-AR agonist, BRL-37344 did not displace the specific binding up to the concentration of 100 μM; the β2-AR antagonist, ICI-1 18551 was effective with a relatively high Ki (28.5 μM) (Table 3). Cyanopindolol was the most effective competitor with a Ki of 0.11 μM, and pindolol had no effect up to the concentration of 100 μM. Carazolol, a ligand structurally related to cyanopindolol, was less effective, in spite of being more lipophilic (Table 3). Interestingly, BRL-35135A (methyl ester of BRL-37344) and ICI 198157 (methyl ester of ICI-201651; ICI-215001, a (S)-enantiomer of ICI-201651) displaced the specific binding, whereas the corresponding acid metabolites were inactive (Table 3). The specific binding was significantly reduced by GTP (29.8 ± 2.7% inhibition at 300 μM ($p < 0.01$) and 98.2 ± 1.3% at 1 mM ($p < 0.001$), n=2, respectively).

TABLE 3

Affinity (Ki) values of various ligands on [^{125}I]-ICYP specific binding to rat colon membranes in the presence of 10 µM 5-HT, 10 µM phentolamine and 20 µM propranolol.

Ligands	Ki (µM)	Pseudo-Hill coefficient
Catecholamines and 5-HT		
(-)-isoproterenol	> 1000	
(-)-norepinephrine	> 1000	
(-)-epinephrin111e	> 1000	
Dopamine	> 1000	
5-HT	> 1000	
SM-11044 and stereo-isomers		
SM-11044 ((l)-threo)	1.8±0.3	1.00±0.12
SM-14786 ((d)-threo)	3.7±0.4	0.92±0.15
SM-14011 ((dl)-threo)	2.0±0.5	1.07±0.15
SM-14010 ((dl)-erythro)	29.3±10.3	0.67±0.13
β1-antagonist		
CGP-20712A	> 100	
β2-antagonist		
ICI-118551	28.5±3.6	0.89±0.14
β3-agonists		
BRL-35135A (ester)	1.4±0.1	0.80±.0.14
BRL-37344 (acid metabolite)	> 100	
ICI-198157 (ester)	29.4±8.9	0.96±0.23
ICI-215001 (acid metabolite)	> 100	
ICI-201651 (acid metabolite)	> 100	
SR-58611A (ester)	5.9±1.0	1.21±0.21
β1-, β2-antagonists having β3-partial agonist potencies		
CGP-12177A	> 100	
(±)-cyanopindolol	0.11±0.02	1.01±0.14
(±)-pindolol	> 100	
(±)-carazolol	8.1±1.7	0.77±0.11
(±)-alprenolol	13.3±2.4	0.85±0.24
β1-, β2-, β3-antagonist		
(±)-bupranolol	11.3±0.8	1.08±0.08

Photoaffinity labeling study

Photoaffinity labeling was performed to visualize the specific binding site in rat colon membranes using [^{125}I]-ICYP-diazirine. In the presence of 10 µM 5-HT and 10 µM phentolamine, but in the absence of propranolol, a single dense band of 34 kDa was visualized in addition to two broad bands with apparent molecular masses of 50 and 70 kDa. (Fig. 14, lane 1). In contrast, in the presence of 20 µM propranolol, 10 µM 5-HT and 10 µM phentolamine, that is, in the same conditions of the competition binding assay with [^{125}I] ICYP, only the 34 kDa band remained visible (Fig. 14, lane 2). These results suggest that the two broad bands are β-ARs. Moreover, the 34 kDa band was not displaced by 100 µM BRL-37344, but was displaced by 100 µM SM-11044 (Fig. 14, lanes 3 and 4, respectively). These data support the results of the competition binding assay, suggesting the existence of a single specific binding site for [^{125}I]-ICYP and SM-11044.

Two-dimensional PAGE of the photoaffinity-labeled membranes confirmed the labeling of a single 34 kDa polypeptide chain corresponding to a pI of 6.0 (Fig. 15). The molecular size of the photoaffinity-labeled 34 kDa protein was not modified by the enzymatic treatments with endoglycosidase or N glycopeptidase F, whereas both enzymes reduced the molecular size of ovalbumin from 43 kDa to 40 kDa. Solubilized photoaffinity-labeled 34 kDa protein (373,298 cpm), were applied to a WGA-sepharose column. The unretained fraction contained 35.7% of the radioactivity, and washed out fractions contained 53.3% of the radioactivity. The specific sugar, 300 mM N-acetyl-D-glucosamine, eluted only 2.3% of the radiolabeled material. The eluted fraction was subjected to SDS-PAGE after concentration, but the photoaffinity-labeled 34 kDa band was not detected. A single 7 kDa labeled-peptide was generated upon digestion of the photoaffinity-labeled 34 kDa protein with trypsin (Fig. 16). Recovery yields in final extracts from the gel pieces were 62.7% for the labeled 34 kDa protein and 90.4% for the in-situ generated tryptic peptides.

Binding studies in rat skeletal muscle membrane preparation

[^{125}I]-ICYP specific binding to skeletal muscle membranes was not displaced by isoproterenol up to concentrations of 10^{-4} M. In contrast, SM-11044 displaced the binding in a concentration-dependent manner (Figure 17).

Pharmacological definition of the instant receptor

- SM-11044, a β-AR agonist, showed atypical agonist effects such as relaxant responses in guinea pig ileum and rat colon intestines, and inhibition of guinea pig eosinophil chemotaxis.

Cyanopindolol competitively antagonized the responses to isoproterenol and SM-11044 at β3-AR with similar pA_2 values (7.32 ~ 7.65) in rat colon intestinal segments and rat white adipocytes. The values were also similar to those reported at β3-AR on rat white adipocytes (Kirkham D. et al., 1992), rat colon, rat gastric fundus (McLaughlin and MacDonald, 1989, 1990), and guinea pig ileum (Blue D.R. et al., 1989). In contrast, cyanopindolol antagonized the additional atypical effect of the SM-11044-induced colon relaxation with higher pA_2 value (8.31) along with low slope of Schild plots (0.78). The results demonstrated the existence of at least two different affinity sites including β3-AR in rat colon. Thus, cyanopindolol and SM-11044 competed not only at β3-AR but also at another atypical binding site. SM-11044 stimulated relaxant responses of the KCl-induced depolarized colon tonus through both sites.

Initial comparison with atypical effects between guinea pig ileum and rat white adipocytes could not exclude species-related difference. However, the difference of atypical effects between rat white adipocytes and rat colon intestines are now evident, that is not species-related phenomenon.

- Detection of the binding site: radioligand binding assay was performed using rat colon smooth muscle membranes based on the results in functional studies that SM-11044 and cyanopindolol competed the sites. In general, if same origin of ligands are used for both radioligand and «cold» ligand, physically- or chemically-related non-specific binding can not be excluded. Furthermore, pA_2 value of cyanopindolol was 8.31 and pD_2 value of SM-11044 was 7.29 in rat colon, suggesting 10-fold higher affinity of cyano-

pindolol than that of SM-11044 at the two atypical components (β3-AR and another site). Therefore, [^{125}I]-ICYP and SM-11044 were used as radioligand and «cold» ligand, respectively.

[^{125}I]-ICYP can bind to β1-, β2-, β3-ARs, serotonine 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors (Tate K.M. et al., 1991; Hoyer D. et al., 1994). In contrast, specific binding was obtained under blockade of these known receptors. Competition binding studies revealed that the binding site was indeed different from these receptors. Natural AR ligands (epinephrine and norepinephrine) and classical β-AR ligand (isoproterenol) showed no affinity, suggesting that the binding site is different from ARs. Several synthetic β-AR ligands including β3-AR agonists (BRL-35135A, SR-58611A and ICI-198157) showed affinity. Atypical effects that could not be explained by β3-AR can be resolved by the existence of this binding site. Indeed, similar binding sites under blockade of β-ARs and serotonine receptors were observed in rat skeletal muscle membranes.

- Biochemical characterization by photoaffinity-labeling study

The binding site in rat colon smooth muscle membranes was visualized by [^{125}I]-ICYP-diazirine, a photoaffinity ligand corresponding to [^{125}I]-ICYP. The apparent molecular size of the site was 34 kDa with an isoelectric point (pI) of 6.0. Deduced molecular sizes of rat β-ARs, serotonine 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors are 43.2 ~ 50.5 kDa (β1-AR, 50.5 kDa; β2-AR, 46.9 kDa; β3-AR, 43.2 kDa; 5-HT$_{1A}$, 46.4 kDa, 5-HT$_{1B}$, 43.2 kDa) (Machida et al., 1990; Gocayne et al., 1987; Muzzin P. et al., 1991; Granneman JG. et al., 1991; Albert A. et al., 1990; Fujiwara et al., 1990, Voigt et al., 1991). In cells or tissues, these receptors are normally glycosylated, then the sizes are usually bigger than the deduced sizes. In contrast, the size of 34 kDa seemed to be smaller than these cloned rat receptors. One explanation may be devoid of N-linked glycosylation. The isoelectric point indicates that the binding site is an acidic protein like β-ARs (Fraser C.M., 1984). Chemical cleavage at mostly methionine residues resulted in 10 and 12 kDa, and acid cleavage at mainly asparagine-proline bonds resulted in 8 kDa, indicating this protein contains methionine residues and may include asparagine-proline bonds.

Example 3: Isolation and characterization of the instant receptor in human skeletal muscle.

- Preparation of probes:

SEQ ID NO:6 has been compared to GenBank and EMBL data base by tblastn program (Altschul S.F. et al., 1990); in dbest data base, a human expressed sequence tag (EST) with almost 100% homology with SEQ ID NO:6 was found; it corresponds to SEQ ID NO:5, found in *H. sapiens* as a partial cDNA sequence, clone 72F05, translated in frame 1 in the form of SEQ ID NO:5. However, it was not known whether or not said SEQ ID NO:5 could have any biological function..

In view to obtain the instant non-adrenergic receptor including SEQ ID NO:1 or NO:13, plasmid DNA containing human clone designated 72F05 (EMBL accession n° z28655) (Auffray C. et al., 1995), including the corresponding coding sequence of SEQ ID NO:5 was obtained from Genethon, France and was used for preparing probes useful for hybridization assays.

900 bp probe (SEQ ID NO:3):

Cutting said plasmid DNA with restriction endonuclease EcoRI (New England Biolabs ref. 101 S) released a 0.9 kb insert corresponding to clone 72F05. This fragment was isolated using QiaEX II agarose gel extraction kit (Qiagen ref. 20021).

300 bp probe (SEQ ID NO:4):

1) Design of sens and anti-sens primers for PCR:

sens primer: S4 (SEQ ID NO:7)

anti-sens primer: S6 (SEQ ID NO:8).

2) PCR on clone 72F05:

Amplification was performed on 1 ng of plasmid DNA corresponding to clone 72F05, in the presence of the following reagents: each primer at 0.25 µM; 10% DMSO; 2.5 U of Taq polymerase (Promega); 0.25 mM of dNTP (dATP; dCTP; dGTP; dTTP); reaction buffer was supplied by Promega and supplemented with 1.5 mM $MgCl_2$.

PCR was performed on Perkin Elmer « Gene Amp PCR System 9600 »

using the following conditions:

4 min at 95°C		
---		
30 sec at 95°C	}	
30 sec at 48°C	}	30 cycles
30 sec at 72°C	}	
---		
4 min at 72°C		

Under these conditions, a 0.3 kb fragment corresponding to the published sequence of clone 72F05 was amplified. The fragment was isolated using QiaEXII agarose gel extraction kit (Qiagen ref. 20021).

- Radiolabeling of probes:

By random priming (Feinberg et al., 1983) 50 µCi of dATP α32P (ICN ref. 39010 X) were incorporated to radiolabel DNA fragments.

- Northern blot:

A human multiple tissue northern blot was purchased from Clontech (ref. 7765-1).

This blot ready to hybridize contained in each lane approximatively 2 µg of polyadenylated mRNA from 8 different human muscles (smooth and striated):

lanes 1-8 in order: human skeletal muscle, uterus (no endometrium), colon (no mucosa), small intestine, bladder, heart, stomach, and prostate (see figure 18A).

The membrane was hybridized following the suppliers instructions with labeled 300 bp probe (SEQ ID NO:4) (10^6 cpm/ml) during 24 hours.

Washes were carried out under different stringency:

1) low stringency: 2x SSC; 0.05% S.D.S. at room temperature.

Exposition of Amersham Hyperfilm MP at -80°C for 3 days using two intensifying screens showed three different fragments: 2 major bands are present in all samples; one at 3.4 kb and one at 3.8 kb. One fainter band, around 7 kb is found in all samples.

2) high stringency: 0.1 x SSC; 0.05% S.D.S. at 50°C; same exposition showed the same fragments in all samples.

The results are illustrated in figure 18 (same results with low or high stringency).

The visualized mRNAs correspond to SMBP transcripts. One explanation for the presence of 3 different transcripts could possibly be the utilization of alternative polyadenylation sites on SMBP gene (Intervening Sequences in Evolution and Development; E.M. Stone and R.J. Schwartz Oxford University Press 1990).

A similar analysis done with a variety of non muscular tissues (heart, brain, placenta, lung, liver, kidney and pancreas) confirmed these observations (figure 18B).

- Cloning of human cDNA:

A human skeletal muscle cDNA library was purchased from Clontech (ref. HL 300s; lot 32288). 500,000 clones were transferred to nylon membranes (Hybond N+; Amersham) and screened by hybridizing either with probe 300 bp (SEQ ID NO:4) or with probe 900 bp (SEQ ID NO:3).

Hybridization conditions were:

600 mM NaCl; 60 mM Na-citrate; 8 mM Tris-HCl pH 7,5; 50 mM Na-phosphate; 1% Ficoll; 1% polyvinylpyrrolidone; 1% bovine serum albumine; 40% formamide; 0.2% SDS; 50 µg/ml salmon sperm DNA.

Radiolabeled probe was added at 10^6 cpm/ml and incubated overnight at 42°C.

Final washes were at 50°C; 0.1 x SSC; 0.05% SDS for 1 hour.

11 positive clones were identified by repeated rounds of screening.

Insert sizes were analyzed by simultaneous cutting with the following restriction endonucleases: Xba I/Hind III and Xba I/Bam HI (New England Biolabs). These enzymes released cDNA inserts from the vector pcDNA I (Invitrogen). All clones were sequenced with T7 and SP6 primers from both ends and found to be overlapping.

The longest cDNA insert (clones n°24 and n°15) was about 1.7 kb and the smallest was about 0.65 kb (clone n°2).

Clone 24 was sequenced on both strands using T7 and SP6 primers and the following specific primers:

Plus strand primers:

S4: SEQ ID NO:7

S6: SEQ ID NO:8

S8: SEQ ID NO: 9

Minus strand primers:

S5: SEQ ID NO:10

S7: SEQ ID NO:11

S9: SEQ ID NO:12.

DNA sequencing data showed a continuous open reading frame (SEQ ID NO:2 or NO:14); translation into protein sequence (SEQ ID NO:1 or NO:13) showed several hydrophobic stretches (figure 23), suggesting that these regions are putative membrane spanning parts of the protein. The sequences corresponding to said hydrophobic stretches are highlighted (boxes) in figure 24.

SMBP appears to share structural homologies with members of a group of proteins described as « similar » to *Saccharomyces cerevisiae* EMP 70 protein precursor.

Figure 22 shows that:

- human myeloblastic cell line D87444 (Nagase T. et al., DNA Res., 1996, **3**, 321-329) is 30% homologous to SMBP,

- p76 protein (Schimmöller F. et al., accession number U81006) is 27% homologous to SMBP,

- the yeast endomembrane protein (Emp70) which is a precursor of a 24 kDa protein (Emp24) involved in intracellular vesicular trafficking (Schimmöller F. et al., EMBO J., 1995, **14**, 7, 1329-1339) is 23% homologous to SMBP,

- hMP70 (Chluba-de Tapia J. et al., Gene, 1997, 197, 195-204) is 28,5% homologous to SMBP whereas

- a protein from *Arabidopsis thaliana* (accession number U95973) is 51,2% homologous to SMDP.

The hydropathy plot of SMBP bears remarkable similarities to those of p76 protein, the myeloblast derived protein, hMP70 protein, *Arabidobsis* protein and Emp70 protein (see figure 23).

The affinity-labeled peptide sequence is located at the switch region between the hydrophobic N-terminal part of SMBP and the C-terminal hydrophobic stretch which contains the transmembrane regions.

The absence of N-glycosylation sites, the lack of homology with plasma membrane receptors and the similarity to intracellular proteins suggest that SMBP could indeed also be an intracellular membrane protein. SMBP appears to be expressed in many different tissues, and could therefore play a major role in normal cellular function. Since SMBP appears to be quite homologous to at least Emp70, involved in intracellular trafficking, i.e. ER via Golgi apparatus; this could also be a role for SMBP.

Example 4: Construction of a plasmid for the expression of Hu-SMBP.

For *in vitro* expression in mammalian cells, 1.7 kb cDNA insert of clone 24 was subcloned into the mammalian expression vector pcDNA3 (Invitrogen). Simultaneous cutting by restriction endonuclease Xba I and Hind III (New England Biolabs) released the 1.7 kb insert from the pcDNA I vector (see example 3). The fragment was then blunt ended using Klenow fragment (Maniatis et al., Molecular Cloning, 2nd edition, 1, 5.40) and purified on 0.7% agarose gel using QiaEX II agarose gel extraction kit (Qiagen ref. 20021).

Vector pcDNA3 was cut in the multisite linker by Eco RV (New England Biolabs) and dephosphorylated using calf intestinal alkaline phosphatase (New England Biolabs). After heat inactivation of phosphatase, the vector and the insert were ligated using T4 DNA ligase (New England Biolabs). Subclone 3 was selected (designated as clone n°24.3). This plasmid contains at least SEQ ID NO:2.

Said recombinant plasmid may be transfected into mammalian cell lines for *in vitro* expression.

Example 5: Expression of SMBP in COS cells.

COS cells were transiently transfected with a vector containing the SMBP nucleotide sequence. The antibodies raised against a synthetic peptide (α8 antibodies) corresponding to the affinity-labeled fragment of rat SMBP were used for immunoprecipitation of proteins extracted from COS cells transfected with the human SMBP cDNA and labeled by I^{125}iodine using the chloramine T procedure. The precipitate was then redissolved and submitted to SDS-PAGE. A single protein with an apparent molecular weight of 45 kDa was identified after autoradiography (figure 25).

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As emerges from the foregoing, the invention is no way limited to those of its embodiments and modes if implementation and application which have just been described more explicitly; it encompasses, on the contrary, all variants which may occur to the specialist in the field, without departure from the scope or range of the present invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: VETIGEN
(B) STREET: 21 rue Sébastien Mercier
(C) CITY: PARIS
(E) COUNTRY: FRANCE
(F) POSTAL CODE (ZIP): 75015

(A) NAME: LENZEN Gerlinde
(B) STREET: 55 rue des Cévennes
(C) CITY: PARIS
(E) COUNTRY: FRANCE
(F) POSTAL CODE (ZIP): 75015

(A) NAME: STROSBERG Arthur Donny
(B) STREET: 66 rue de Javel
(C) CITY: PARIS
(E) COUNTRY: FRANCE
(F) POSTAL CODE (ZIP): 75015

(A) NAME: SUGASAWA Toshinari
(B) STREET: 9-12-507 Miyano-cho, Takatsuki-shi
(C) CITY: OSAKA
(E) COUNTRY: JAPON
(F) POSTAL CODE (ZIP): 569

(A) NAME: MOROOKA Shigeako
(B) STREET: 4-78 nishi-3, Seiwadai, Kawanishi-shi
(C) CITY: HYOGO
(E) COUNTRY: JAPON
(F) POSTAL CODE (ZIP): 666-01

(ii) TITLE OF INVENTION: NON-ADRENERGIC RECEPTOR AND ITS APPLICATIONS.

(iii) NUMBER OF SEQUENCES: 14

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 439 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Met Tyr Ile Asp Asp Leu Pro Ile Trp Gly Ile Val Gly Glu Ala Asp
1               5                  10                  15

Glu Asn Gly Glu Asp Tyr Tyr Leu Trp Thr Tyr Lys Lys Leu Glu Ile
            20                  25                  30

Gly Phe Asn Gly Asn Arg Ile Val Asp Val Asn Leu Thr Ser Glu Gly
        35                  40                  45
```

Lys Val Lys Leu Val Pro Asn Thr Lys Ile Gln Met Ser Tyr Ser Val
50 55 60

Lys Trp Lys Lys Ser Asp Val Lys Phe Glu Asp Arg Phe Asp Lys Tyr
65 70 75 80

Leu Asp Pro Ser Phe Phe Gln His Arg Ile His Trp Phe Ser Ile Phe
85 90 95

Asn Ser Phe Met Met Val Ile Phe Leu Val Gly Leu Val Ser Met Ile
100 105 110

Leu Met Arg Thr Leu Arg Lys Asp Tyr Ala Arg Tyr Ser Lys Glu Glu
115 120 125

Glu Met Asp Asp Met Asp Arg Asp Leu Gly Asp Glu Tyr Gly Trp Lys
130 135 140

Gln Val His Gly Asp Val Phe Arg Pro Ser Ser His Pro Leu Ile Phe
145 150 155 160

Ser Ser Leu Ile Gly Ser Gly Cys Gln Ile Phe Ala Val Ser Leu Ile
165 170 175

Val Ile Ile Val Ala Met Ile Glu Asp Leu Tyr Thr Glu Arg Gly Ser
180 185 190

Met Leu Ser Thr Ala Ile Phe Val Tyr Ala Ala Thr Ser Pro Val Asn
195 200 205

Gly Tyr Phe Gly Gly Ser Leu Tyr Ala Arg Gln Gly Gly Arg Arg Trp
210 215 220

Ile Lys Gln Met Phe Ile Gly Ala Phe Leu Ile Pro Ala Met Val Cys
225 230 235 240

Gly Thr Ala Phe Phe Ile Asn Phe Ile Ala Ile Tyr Tyr His Ala Ser
245 250 255

Arg Ala Ile Pro Phe Gly Thr Met Val Ala Val Cys Cys Ile Cys Phe
260 265 270

Phe Val Ile Leu Pro Leu Asn Leu Val Gly Thr Ile Leu Gly Arg Asn
275 280 285

Leu Ser Gly Gln Pro Asn Phe Pro Cys Arg Val Asn Ala Val Pro Arg
290 295 300

Pro Ile Pro Glu Lys Lys Trp Phe Met Glu Pro Ala Val Ile Val Cys
305 310 315 320

Leu Gly Gly Ile Leu Pro Phe Gly Ser Ile Phe Ile Glu Met Tyr Phe
325 330 335

Ile Phe Thr Ser Phe Trp Ala Tyr Lys Ile Tyr Tyr Val Tyr Gly Phe
340 345 350

Met Met Leu Val Leu Val Ile Leu Cys Ile Val Thr Val Cys Val Thr
355 360 365

Ile Val Cys Thr Tyr Phe Leu Leu Asn Ala Glu Asp Tyr Arg Trp Gln
370 375 380

Trp Thr Ser Phe Leu Ser Ala Ala Ser Thr Ala Ile Tyr Val Tyr Met
385 390 395 400

Tyr Ser Phe Tyr Tyr Tyr Phe Phe Lys Thr Lys Met Tyr Gly Leu Phe
405 410 415

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Gln Thr Ser Phe Tyr Phe Gly Tyr Met Ala Val Phe Ser Thr Ala Leu
420 425 430

Gly Ile Met Cys Gly Ala Ile
435

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1317 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATGTACATAG ATGATTTACC AATATGGGGT ATTGTTGGTG AGGCTGATGA AAATGGAGAA 60

GATTACTATC TTTGGACCTA TAAAAAACTT GAAATAGGTT TTAATGGAAA TCGAATTGTT 120

GATGTTAATC TAACTAGTGA AGGAAAGGTG AAACTGGTTC CAAATACTAA AATCCAGATG 180

TCATATTCAG TAAAATGGAA AAAGTCAGAT GTGAAATTTG AAGATCGATT TGACAAATAT 240

CTTGATCCGT CCTTTTTTCA ACATCGGATT CATTGGTTTT CAATTTTCAA CTCCTTCATG 300

ATGGTGATCT TCTTGGTGGG CTTAGTTTCA ATGATTTTAA TGAGAACATT AAGAAAAGAT 360

TATGCTCGGT ACAGTAAAGA GGAAGAAATG GATGATATGG ATAGAGACCT AGGAGATGAA 420

TATGGATGGA AACAGGTGCA TGGAGATGTA TTTAGACCAT CAAGTCACCC ACTGATATTT 480

TCCTCTCTGA TTGGTTCTGG ATGTCAGATA TTTGCTGTGT CTCTCATCGT TATTATTGTT 540

GCAATGATAG AAGATTTATA TACTGAGAGG GGATCAATGC TCAGTACAGC CATATTTGTC 600

TATGCTGCTA CGTCTCCAGT GAATGGTTAT TTTGGAGGAA GTCTGTATGC TAGACAAGGA 660

GGAAGGAGAT GGATAAAGCA GATGTTTATT GGGGCATTCC TTATCCCAGC TATGGTGTGT 720

GGCACTGCCT TCTTCATCAA TTTCATAGCC ATTTATTACC ATGCTTCAAG AGCCATTCCT 780

TTTGGAACAA TGGTGGCCGT TTGTTGCATC TGTTTTTTTG TTATTCTTCC TCTAAATCTT 840

GTTGGTACAA TACTTGGCCG AAATCTGTCA GGTCAGCCCA ACTTTCCTTG TCGTGTCAAT 900

GCTGTGCCTC GTCCTATACC GGAGAAAAAA TGGTTCATGG AGCCTGCGGT TATTGTTTGC 960

CTGGGTGGAA TTTTACCTTT TGGTTCAATC TTTATTGAAA TGTATTTCAT CTTCACGTCT 1020

TTCTGGGCAT ATAAGATCTA TTATGTCTAT GGCTTCATGA TGCTGGTGCT GGTTATCCTG 1080

TGCATTGTGA CTGTCTGTGT GACTATTGTG TGCACATATT TTCTACTAAA TGCAGAAGAT 1140

TACCGGTGGC AATGGACAAG TTTTCTCTCT GCTGCATCAA CTGCAATCTA TGTTTACATG 1200

TATTCCTTTT ACTACTATTT TTTCAAAACA AAGATGTATG GCTTATTTCA AACATCATTT 1260

TACTTTGGAT ATATGGCGGT ATTTAGCACA GCCTTGGGGA TAATGTGTGG AGCGATT 1317

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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 965 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CAGATGTCAT ATTCAGTAAA ATGGAAAAAG TCAGATGTGA AATTTGAAGA TCGATTTGAC 60

AAATATCTTG ATCCGTCCTT TTTTCAACAT CGGATTCATT GGTTTTCAAT TTTCAACTCC 120

TTCATGATGG TGATCTTCTT GGTGGGCTTA GTTTCAATGA TTTTAATGAG AACATTAAGA 180

AAAGATTATG CTCGGTACAG TAAAGAGGAA GAAATGGATG ATATGGATAG AGACCTAGGA 240

GATGAATATG GATGGAAACA GGTGCATGGA GATGTATTTA GACCATCAAG TCACCCACTG 300

ATATTTTCCT CTCTGATTGG TTCTGGATGT CAGATATTTG CTGTGTCTCT CATCGTTATT 360

ATTGTTGCAA TGATAGAAGA TTTATATACT GAGAGGGGAT CAATGCTCAG TACAGCCATA 420

TTTGTCTATG CTGCTACGTC TCCAGTGAAT GGTTATTTTA GAGGAAGTCT GTATGCTAGA 480

CAAGGAGGAA GGAGATGGAT AAAGCAGATG TTTATTGGGG CATTCCTTAT CCCAGCTATG 540

GTGTGTGGCA CTGCCTTCTT CATCAATTTC ATAGCCATTT ATTACCATGC TTCAAGAGCC 600

ATTCCTTTTG GAACAATGGT GGCCGTTTGT TGCATCTGTT TTTTTGTTAT TCTTCCTCTA 660

AATCTTGTTG GTACAATACT TGGCCGAAAT CTGTCAGGTC AGCCCAACTT TCCTTGTCGT 720

GTCAATGCTG TGCCTCGTCC TATACCGGAG AAAAAATGGT TCATGGAGCC TGCGGTTATT 780

GTTTGCCTGG GTGGAATTTT ACCTTTTGGT TCAATCTTTA TTGAAATGTA TTTCATCTTC 840

ACGTCTTTCT GGGCATATAA GATCTATTAT GTCTATGGCT TCATGATGCT GGTGCTGGTT 900

ATCCTGTGCA TTGTGACTGT CTGTGTGACT ATTGTGTGCA CATATTTTCT ACTAAATGCA 960

GAAGA 965

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 285 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TCAGTAAAAT GGAAAAAGTC AGATGTGAAA TTTGAAGATC GATTTGACAA ATATCTTGAT 60

CCGTCCTTTT TTCAACATCG GATTCATTGG TTTTCAATTT TCAACTCCTT CATGATGGTG 120

ATCTTCTTGG TGGGCTTAGT TTCAATGATT TTAATGAGAA CATTAAGAAA AGATTATGCT 180

CGGTACAGTA AAGAGGAAGA AATGGATGAT ATGGATAGAG ACCTAGGAGA TGAATATGGA 240

TGGAAACAGG TGCATGGAGA TGTATTTAGA CCATCAAGTC ACCCA 285

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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Asp Pro Ser Phe Phe Gln His Arg Ile His Trp Phe Ser Ile Phe Asn
1 5 10 15

Ser

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Asp Pro Xaa Phe Phe Gln His Arg Ile His Val Phe Ser Ile Phe Asn
1 5 10 15

His

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "AMORCE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TCAGTAAAAT GGAAAAAGTC 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "AMORCE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TGGGTGACTT GATGGTCTAA 20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "AMORCE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GCTGTGTCTC TCATCGTTA 19

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "AMORCE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CCATCCATAT TCATCTCCTA 20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "AMORCE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CGGTATAGGA CGAGGCACAG C 21

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "AMORCE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

ACTGAATATG ACATCTGG 18

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1800 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:3..1730

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
CC GCC GCG CTG TGG CTG CTG CTG CTG CTG CTG CCC CGG ACC CGG GCG          47
   Ala Ala Leu Trp Leu Leu Leu Leu Leu Leu Pro Arg Thr Arg Ala
     1               5                  10                  15

GAC GAG CAC GAA CAC ACG TAT CAA GAT AAA GAG GAA GTT GTC TTA TGG          95
Asp Glu His Glu His Thr Tyr Gln Asp Lys Glu Glu Val Val Leu Trp
                 20                  25                  30

ATG AAT ACT GTT GGG CCC TAC CAT AAT CGT CAA GAA ACA TAT AAG TAC         143
Met Asn Thr Val Gly Pro Tyr His Asn Arg Gln Glu Thr Tyr Lys Tyr
             35                  40                  45

TTT TCA CTT CCA TTC TGT GTG GGG TCA AAA AAA AGT ATC AGT CAT TAC         191
Phe Ser Leu Pro Phe Cys Val Gly Ser Lys Lys Ser Ile Ser His Tyr
         50                  55                  60

CAT GAA ACT CTG GGA GAA GCA CTT CAA GGG GTT GAA TTG GAA TTT AGT         239
His Glu Thr Leu Gly Glu Ala Leu Gln Gly Val Glu Leu Glu Phe Ser
     65                  70                  75

GGT CTG GAT ATT AAA TTT AAA GAT GAT GTG ATG CCA GCC ACT TAC TGT         287
Gly Leu Asp Ile Lys Phe Lys Asp Asp Val Met Pro Ala Thr Tyr Cys
 80                  85                  90                  95

GAA ATT GAT TTA GAT AAA GAA AAG AGA GAT GCA TTT GTA TAT GCC ATA         335
Glu Ile Asp Leu Asp Lys Glu Lys Arg Asp Ala Phe Val Tyr Ala Ile
                100                 105                 110

AAA AAT CAT TAC TGG TAC CAG ATG TAC ATA GAT GAT TTA CCA ATA TGG         383
Lys Asn His Tyr Trp Tyr Gln Met Tyr Ile Asp Asp Leu Pro Ile Trp
            115                 120                 125

GGT ATT GTT GGT GAG GCT GAT GAA AAT GGA GAA GAT TAC TAT CTT TGG         431
Gly Ile Val Gly Glu Ala Asp Glu Asn Gly Glu Asp Tyr Tyr Leu Trp
        130                 135                 140

ACC TAT AAA AAA CTT GAA ATA GGT TTT AAT GGA AAT CGA ATT GTT GAT         479
Thr Tyr Lys Lys Leu Glu Ile Gly Phe Asn Gly Asn Arg Ile Val Asp
    145                 150                 155

GTT AAT CTA ACT AGT GAA GGA AAG GTG AAA CTG GTT CCA AAT ACT AAA         527
Val Asn Leu Thr Ser Glu Gly Lys Val Lys Leu Val Pro Asn Thr Lys
160                 165                 170                 175

ATC CAG ATG TCA TAT TCA GTA AAA TGG AAA AAG TCA GAT GTG AAA TTT         575
Ile Gln Met Ser Tyr Ser Val Lys Trp Lys Lys Ser Asp Val Lys Phe
                180                 185                 190

GAA GAT CGA TTT GAC AAA TAT CTT GAT CCG TCC TTT TTT CAA CAT CGG         623
Glu Asp Arg Phe Asp Lys Tyr Leu Asp Pro Ser Phe Phe Gln His Arg
            195                 200                 205
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ATT CAT TGG TTT TCA ATT TTC AAC TCC TTC ATG ATG GTG ATC TTC TTG          671
Ile His Trp Phe Ser Ile Phe Asn Ser Phe Met Met Val Ile Phe Leu
        210                 215                 220

GTG GGC TTA GTT TCA ATG ATT TTA ATG AGA ACA TTA AGA AAA GAT TAT          719
Val Gly Leu Val Ser Met Ile Leu Met Arg Thr Leu Arg Lys Asp Tyr
    225                 230                 235

GCT CGG TAC AGT AAA GAG GAA GAA ATG GAT GAT ATG GAT AGA GAC CTA          767
Ala Arg Tyr Ser Lys Glu Glu Glu Met Asp Asp Met Asp Arg Asp Leu
240                 245                 250                 255

GGA GAT GAA TAT GGA TGG AAA CAG GTG CAT GGA GAT GTA TTT AGA CCA          815
Gly Asp Glu Tyr Gly Trp Lys Gln Val His Gly Asp Val Phe Arg Pro
                260                 265                 270

TCA AGT CAC CCA CTG ATA TTT TCC TCT CTG ATT GGT TCT GGA TGT CAG          863
Ser Ser His Pro Leu Ile Phe Ser Ser Leu Ile Gly Ser Gly Cys Gln
            275                 280                 285

ATA TTT GCT GTG TCT CTC ATC GTT ATT ATT GTT GCA ATG ATA GAA GAT          911
Ile Phe Ala Val Ser Leu Ile Val Ile Ile Val Ala Met Ile Glu Asp
        290                 295                 300

TTA TAT ACT GAG AGG GGA TCA ATG CTC AGT ACA GCC ATA TTT GTC TAT          959
Leu Tyr Thr Glu Arg Gly Ser Met Leu Ser Thr Ala Ile Phe Val Tyr
    305                 310                 315

GCT GCT ACG TCT CCA GTG AAT GGT TAT TTT GGA GGA AGT CTG TAT GCT         1007
Ala Ala Thr Ser Pro Val Asn Gly Tyr Phe Gly Gly Ser Leu Tyr Ala
320                 325                 330                 335

AGA CAA GGA GGA AGG AGA TGG ATA AAG CAG ATG TTT ATT GGG GCA TTC         1055
Arg Gln Gly Gly Arg Arg Trp Ile Lys Gln Met Phe Ile Gly Ala Phe
                340                 345                 350

CTT ATC CCA GCT ATG GTG TGT GGC ACT GCC TTC TTC ATC AAT TTC ATA         1103
Leu Ile Pro Ala Met Val Cys Gly Thr Ala Phe Phe Ile Asn Phe Ile
            355                 360                 365

GCC ATT TAT TAC CAT GCT TCA AGA GCC ATT CCT TTT GGA ACA ATG GTG         1151
Ala Ile Tyr Tyr His Ala Ser Arg Ala Ile Pro Phe Gly Thr Met Val
        370                 375                 380

GCC GTT TGT TGC ATC TGT TTT TTT GTT ATT CTT CCT CTA AAT CTT GTT         1199
Ala Val Cys Cys Ile Cys Phe Phe Val Ile Leu Pro Leu Asn Leu Val
    385                 390                 395

GGT ACA ATA CTT GGC CGA AAT CTG TCA GGT CAG CCC AAC TTT CCT TGT         1247
Gly Thr Ile Leu Gly Arg Asn Leu Ser Gly Gln Pro Asn Phe Pro Cys
400                 405                 410                 415

CGT GTC AAT GCT GTG CCT CGT CCT ATA CCG GAG AAA AAA TGG TTC ATG         1295
Arg Val Asn Ala Val Pro Arg Pro Ile Pro Glu Lys Lys Trp Phe Met
                420                 425                 430

GAG CCT GCG GTT ATT GTT TGC CTG GGT GGA ATT TTA CCT TTT GGT TCA         1343
Glu Pro Ala Val Ile Val Cys Leu Gly Gly Ile Leu Pro Phe Gly Ser
            435                 440                 445

ATC TTT ATT GAA ATG TAT TTC ATC TTC ACG TCT TTC TGG GCA TAT AAG         1391
Ile Phe Ile Glu Met Tyr Phe Ile Phe Thr Ser Phe Trp Ala Tyr Lys
        450                 455                 460

ATC TAT TAT GTC TAT GGC TTC ATG ATG CTG GTG CTG GTT ATC CTG TGC         1439
Ile Tyr Tyr Val Tyr Gly Phe Met Met Leu Val Leu Val Ile Leu Cys
    465                 470                 475
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ATT GTG ACT GTC TGT GTG ACT ATT GTG TGC ACA TAT TTT CTA CTA AAT      1487
Ile Val Thr Val Cys Val Thr Ile Val Cys Thr Tyr Phe Leu Leu Asn
480                 485                 490                 495

GCA GAA GAT TAC CGG TGG CAA TGG ACA AGT TTT CTC TCT GCT GCA TCA      1535
Ala Glu Asp Tyr Arg Trp Gln Trp Thr Ser Phe Leu Ser Ala Ala Ser
                500                 505                 510

ACT GCA ATC TAT GTT TAC ATG TAT TCC TTT TAC TAC TAT TTT TTC AAA      1583
Thr Ala Ile Tyr Val Tyr Met Tyr Ser Phe Tyr Tyr Tyr Phe Phe Lys
            515                 520                 525

ACA AAG ATG TAT GGC TTA TTT CAA ACA TCA TTT TAC TTT GGA TAT ATG      1631
Thr Lys Met Tyr Gly Leu Phe Gln Thr Ser Phe Tyr Phe Gly Tyr Met
        530                 535                 540

GCG GTA TTT AGC ACA GCC TTG GGG ATA ATG TGT GGA GCG ATT GGT TAC      1679
Ala Val Phe Ser Thr Ala Leu Gly Ile Met Cys Gly Ala Ile Gly Tyr
    545                 550                 555

ATG GGA ACA AGT GCC TTT GTC CGA AAA ATC TAT ACT AAT GTG AAA ATT      1727
Met Gly Thr Ser Ala Phe Val Arg Lys Ile Tyr Thr Asn Val Lys Ile
560                 565                 570                 575

GAC TAGAGACCCA AGAAAACCTG GAACTTTGGA TCAATTTCTT TTTCATAGGG           1780
Asp

GTGGAACTTG CACAGCAAAA                                               1800
```

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 576 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

```
Ala Ala Leu Trp Leu Leu Leu Leu Leu Leu Pro Arg Thr Arg Ala Asp
  1               5                  10                  15

Glu His Glu His Thr Tyr Gln Asp Lys Glu Glu Val Val Leu Trp Met
             20                  25                  30

Asn Thr Val Gly Pro Tyr His Asn Arg Gln Glu Thr Tyr Lys Tyr Phe
         35                  40                  45

Ser Leu Pro Phe Cys Val Gly Ser Lys Lys Ser Ile Ser His Tyr His
     50                  55                  60

Glu Thr Leu Gly Glu Ala Leu Gln Gly Val Glu Leu Glu Phe Ser Gly
 65                  70                  75                  80

Leu Asp Ile Lys Phe Lys Asp Asp Val Met Pro Ala Thr Tyr Cys Glu
                 85                  90                  95

Ile Asp Leu Asp Lys Glu Lys Arg Asp Ala Phe Val Tyr Ala Ile Lys
            100                 105                 110

Asn His Tyr Trp Tyr Gln Met Tyr Ile Asp Asp Leu Pro Ile Trp Gly
        115                 120                 125

Ile Val Gly Glu Ala Asp Glu Asn Gly Glu Asp Tyr Tyr Leu Trp Thr
    130                 135                 140

Tyr Lys Lys Leu Glu Ile Gly Phe Asn Gly Asn Arg Ile Val Asp Val
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145 150 155 160

Asn Leu Thr Ser Glu Gly Lys Val Lys Leu Val Pro Asn Thr Lys Ile
165 170 175

Gln Met Ser Tyr Ser Val Lys Trp Lys Lys Ser Asp Val Lys Phe Glu
180 185 190

Asp Arg Phe Asp Lys Tyr Leu Asp Pro Ser Phe Phe Gln His Arg Ile
195 200 205

His Trp Phe Ser Ile Phe Asn Ser Phe Met Met Val Ile Phe Leu Val
210 215 220

Gly Leu Val Ser Met Ile Leu Met Arg Thr Leu Arg Lys Asp Tyr Ala
225 230 235 240

Arg Tyr Ser Lys Glu Glu Glu Met Asp Asp Met Asp Arg Asp Leu Gly
245 250 255

Asp Glu Tyr Gly Trp Lys Gln Val His Gly Asp Val Phe Arg Pro Ser
260 265 270

Ser His Pro Leu Ile Phe Ser Ser Leu Ile Gly Ser Gly Cys Gln Ile
275 280 285

Phe Ala Val Ser Leu Ile Val Ile Ile Val Ala Met Ile Glu Asp Leu
290 295 300

Tyr Thr Glu Arg Gly Ser Met Leu Ser Thr Ala Ile Phe Val Tyr Ala
305 310 315 320

Ala Thr Ser Pro Val Asn Gly Tyr Phe Gly Gly Ser Leu Tyr Ala Arg
325 330 335

Gln Gly Gly Arg Arg Trp Ile Lys Gln Met Phe Ile Gly Ala Phe Leu
340 345 350

Ile Pro Ala Met Val Cys Gly Thr Ala Phe Phe Ile Asn Phe Ile Ala
355 360 365

Ile Tyr Tyr His Ala Ser Arg Ala Ile Pro Phe Gly Thr Met Val Ala
370 375 380

Val Cys Cys Ile Cys Phe Phe Val Ile Leu Pro Leu Asn Leu Val Gly
385 390 395 400

Thr Ile Leu Gly Arg Asn Leu Ser Gly Gln Pro Asn Phe Pro Cys Arg
405 410 415

Val Asn Ala Val Pro Arg Pro Ile Pro Glu Lys Lys Trp Phe Met Glu
420 425 430

Pro Ala Val Ile Val Cys Leu Gly Gly Ile Leu Pro Phe Gly Ser Ile
435 440 445

Phe Ile Glu Met Tyr Phe Ile Phe Thr Ser Phe Trp Ala Tyr Lys Ile
450 455 460

Tyr Tyr Val Tyr Gly Phe Met Met Leu Val Leu Val Ile Leu Cys Ile
465 470 475 480

Val Thr Val Cys Val Thr Ile Val Cys Thr Tyr Phe Leu Leu Asn Ala
485 490 495

Glu Asp Tyr Arg Trp Gln Trp Thr Ser Phe Leu Ser Ala Ala Ser Thr
500 505 510

Ala Ile Tyr Val Tyr Met Tyr Ser Phe Tyr Tyr Tyr Phe Phe Lys Thr

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515 520 525

Lys Met Tyr Gly Leu Phe Gln Thr Ser Phe Tyr Phe Gly Tyr Met Ala
530 535 540

Val Phe Ser Thr Ala Leu Gly Ile Met Cys Gly Ala Ile Gly Tyr Met
545 550 555 560

Gly Thr Ser Ala Phe Val Arg Lys Ile Tyr Thr Asn Val Lys Ile Asp
565 570 575

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MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 5, line 25 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☐

Name of depositary institution

Collection Nationale de Cultures de Micro-organismes

Address of depositary institution (including postal code and country)

28 rue du Docteur Roux, 75724 PARIS CEDEX 15

Date of deposit	Accession Number
December 10, 1996	I-1795

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet ☐

"With regard to the nominations in which a European patent is applied for, until the publication of the mention of the grant of the European patent or until the date on which the application shall be refused or withdrawn or shall be deemed to be withdrawn, a sample of the deposited microorganism shall be available only by the issue of a sample to an expert nominated by the requester. (Rule 28.4) of the EPC)".

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

EUROPE
CANADA
JAPAN
UNITED STATES OF AMERICA

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

(illegible signature)
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau was

(Authorized Officer)

(January 1985)

CLAIMS

1°) Substantially pure mammal polypeptide, characterized in that it contains sites such that when said sites are exposed at the surface of a cell, they are able of binding iodocyanopindolol (ICYP) under blockade of α, β1, β2, β3-AR, serotonine 5-HT_{1A} ~~and~~ serotonine 5-HT_{1B} receptors, said binding being saturable, reversible, able to be displaced by a β-adrenergic receptor agonist SM-11044 with stereoselectivity but not by isoproterenol, norepinephrine, epinephrine, serotonine, dopamine or BRL-37344, and not being blocked by propranolol, said polypeptide (1) having an apparent molecular weight of about 30-40 kDa when labeled with ^{125}I-iodocyanopindolol after photoaffinity labeling and separation by electrophoresis and an apparent molecular weight of about 60-80 kDa in Western blot, and (2) generating a fragment having the following formula $DPX_1FFQHRIHX_2FSIFNX_3$ by acidic cleavage, wherein, X_1 represents S (SEQ ID N°5) or X (SEQ ID N°6), X_2 represents V (SEQ ID N°6) or W (SEQ ID N°5) and X_3 represents S (SEQ ID N°5) or H (SEQ ID N°6), said polypeptide being present at least on muscles and eosinophils membranes and being a non-adrenergic receptor.

2°) The polypeptide according to claim 1, characterized in that it contains at least SEQ ID NO:1.

3°) The polypeptide according to claim 1, characterized in that it consists of SEQ ID NO:13.

4°) An isolated and purified nucleic acid sequence, characterized in that it encodes a mammalian receptor as claimed in claim 1.

5°) The isolated and purified nucleic acid sequence of claim 4, characterized in that it includes at least SEQ ID NO:2.

6°) The isolated and purified nucleic acid sequence of claim 4, characterized in that it consists of SEQ ID NO:14.

7°) The purified nucleic acid sequence according to claim 4 or claim 5, characterized in that it hybridizes with SEQ ID NO:3 or SEQ ID NO:4.

8°) cDNA clones, characterized in that they comprise a sequence coding for the instant non-adrenergic receptor according to claim 1.

9°) Synthetic or non-synthetic nucleotide probes, characterized in that they hybridize with a nucleic acid according to claims 4 to 8 or with their complementary sequences or their corresponding RNA, these probes being such that they do not hybridize with the genes or the messenger RNA coding for β-adrenergic receptors.

10°) Probes according to claim 9, characterized in that they are selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:7 to SEQ ID NO:12, optionally labeled using a label such as a radioactive isotope, a suitable enzyme or a fluorochrome.

11°) Primers for amplifying a nucleic acid sequence according to claims 4 to 8, characterized in that they are selected from the group consisting of SEQ ID NO:7 to SEQ ID NO:12.

12°) A recombinant plasmid in particular for cloning and/or expression, containing a nucleic acid sequence according to claims 4 to 8 at one of its cloning sites non essential for its replication.

13°) The recombinant plasmid according to claim 12, characterized in that it further comprises an origin of replication for replication in a host cell, at least one gene whose expression permits selection of said host cell transformed with said plasmid, and a regulatory sequence, including a promoter permitting expression of a polypeptide having a non-adrenergic activity as defined hereabove, in said host cell.

14°) The recombinant plasmid according to claim 12, characterized in that said plasmid is pcDNA3 into which is inserted, in a multisite linker. SEQ ID NO:2, wherein said plasmid is deposited with the Collection Nationale de Cultures de Microorganismes [National Collection of Microorganism Cultures] (CNCM held by the PASTEUR INSTITUTE, dated December 10, 1996, under No. I-1795.

15°) A host cell transformed by a recombinant plasmid according to claims 12 to 14, characterized in that it comprises the elements of regulation making possible the expression of the nucleotide sequence coding for the instant polypeptide in this host.

16°) The host cell according to claim 15, characterized in that it consists, in particular, in mammalian cell lines.

17°) Antibodies directed specifically against the instant polypeptide according to claim 1, these antibodies being such that they recognize neither known α or β–adrenergic, nor serotonine, nor dopamine receptors.

18°) A method for assaying a substance for agonist or antagonist activity towards a polypeptide according to claim 1, which method comprises:

- placing the substance in contact with tissue membrane proteins or a transformed host cell expressing a polypeptide according to claim 1 under conditions which permit binding between said polypeptide binding sites and an agonist or an antagonist thereto and

- measuring an appropriate transduction signal.

19°) A process for studying the binding affinity of a compound for a polypeptide according to claim 1, which process comprises:

- transforming a host cell by an expression vector comprising a nucleotide sequence coding for the instant receptor,

- culturing said transformed host cell under conditions which permit the expression or said receptor encoded by said nucleotide sequence and the transfer of the expressed receptor polypeptide to the membrane of the said transformed host cell so that transmembrane sequences of said receptor polypeptide are embedded in the cell membranes of the transformed host cell;

- placing said transformed host cell in contact with said compound and

- measuring the quantity of said compound bond to said receptor polypeptide.

20°) A process for studying the binding affinity of a compound for a polypeptide according to claim 1, which process comprises:

- extracting membrane proteins corresponding to the instant receptor polypeptide from appropriate tissues or cells such as muscles,

- placing said membrane proteins in contact with said compound and

- measuring the quantity of said compound bond to said receptor polypeptide.

21°) Method of preparation of a polypeptide according to claim 1, which method comprises:

- preparing membrane proteins from a tissue containing the instant polypeptide, such as rat colon or human skeletal muscle,

- labeling said membrane proteins with [^{125}I]-ICYP-diazirine or another appropriate marker under blockade of α, β1, β2, β3-AR and serotonine receptors,

- separating said labeled proteins by preparative SDS-PAGE electrophoresis and

- extracting the radioactive band.

add Cl 17

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International Bureau

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(71) Applicant *(for all designated States except US)*: VETIGEN [FR/FR]; 21, rue Sébastien Mercier, F–75015 Paris (FR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: LENZEN, Gerlinde [DE/FR]; 55, rue des Cévennes, F–75015 Paris (FR). STROSBERG, Arthur, Donny [BE/FR]; 66, rue de Javel, F–75015 Paris (FR). SUGASAWA, Toshinari [JP/JP]; 9–12–507, Miyano–cho, Takatsuki–shi, Osaka 569 (JP). MOROOKA, Shigeaki [JP/JP]; 4–78, Nishi–3, Seiwadai, Kawanishi–shi, Hyogo 666–01 (JP).

(74) Agent: CABINET ORES; 6, avenue de Messine, F–75008 Paris (FR).

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(54) Title: MAMMALIAN ICYP (IODOCYANOPINDOLOL) RECEPTOR AND ITS APPLICATIONS

(57) Abstract

An isolated and substantially pure mammal polypeptide different from known adrenergic, serotonine and dopamine receptors, existing at least on mammalian muscle and eosinophils membranes, for instance in rat, guinea pig and humans. The invention also relates to plasmids containing the genes coding for said polypeptide, to host cells transformed by genes coding for the above mentioned polypeptide, to nucleotide probes capable of hybridizing with the genes coding for the above mentioned polypeptide, and to polyclonal and monoclonal antibodies directed against the above mentioned polypeptide. Said polypeptide is characterized in that it contains sites such that when said sites are exposed at the surface of a cell, they are able of binding iodocyanopindolol (ICYP) under blockage of α, $\beta 1$, $\beta 2$, $\beta 3$–AR, serotonine 5–HT_{1A} and serotonine 5–HT_{1B} receptors, said binding being saturable, reversible, able to be displaced by a β–adrenergic receptor agonist SM–11044 with stereoselectivity but not by isoproterenol, norepi–nephrine, epinephrine, serotonine, dopamine or BRL–37344, and not being blocked by propranolol, said polypeptide (1) having an apparent molecular weight of about 30–40 kDa when labeled with ^{125}I–iodocyanopindolol after photoaffinity labeling and separation by electrophoresis and an apparent molecular weight of about 60–80 kDa in Western blot, and (2) generating a fragment having the following formula $DPX_1FFQHRIHX_2FSIFNX_3$ by acidic cleavage, wherein X_1 represents S (SEQ ID NO.5) or X (SEQ ID NO.6), X_2 represents V (SEQ ID NO.6) or W (SEQ ID NO.5) and X_3 represents S (SEQ ID NO.5) or H (SEQ ID NO.6), said polypeptide being present at least on muscles and eosinophils membranes and being a non–adrenergic receptor.

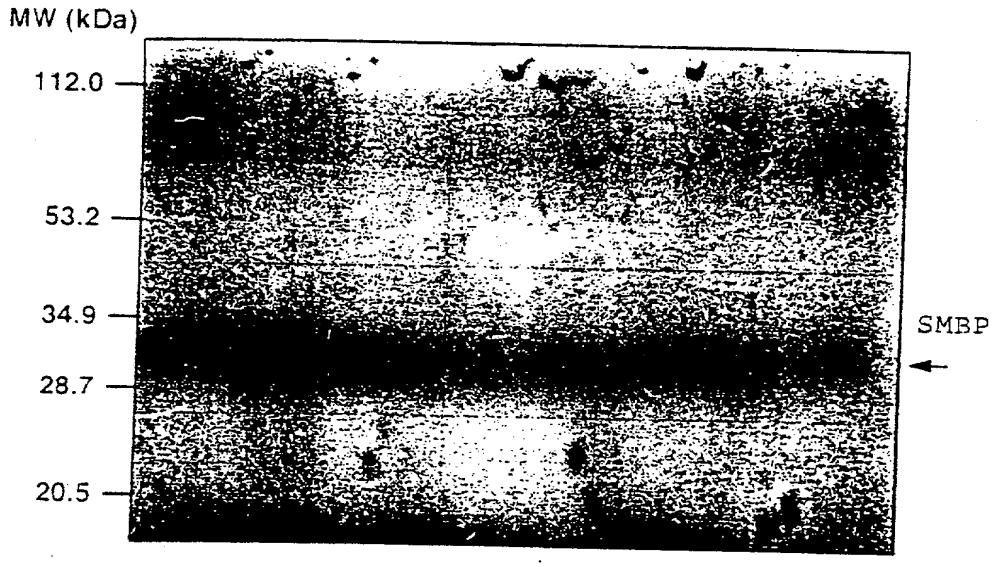


FIGURE 1

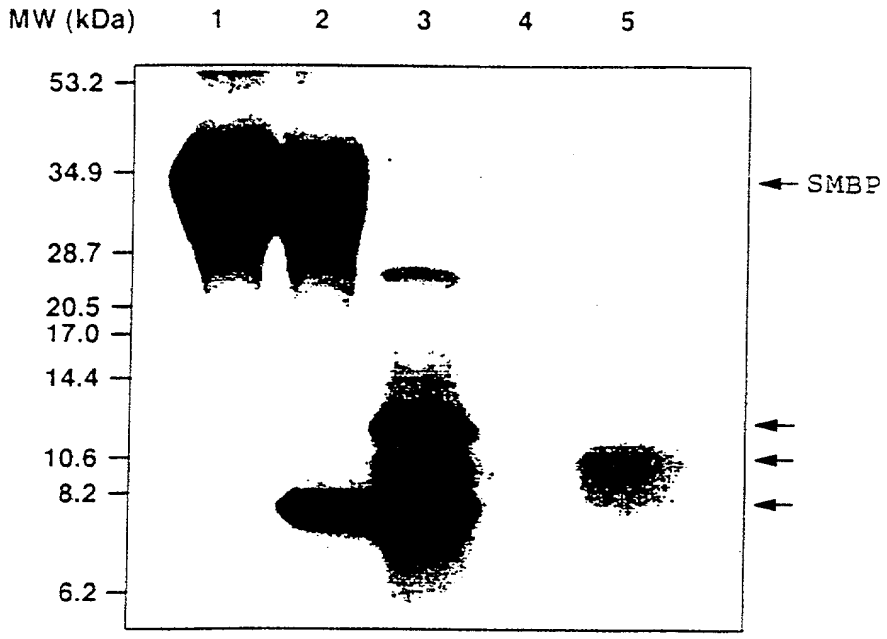


FIGURE 2

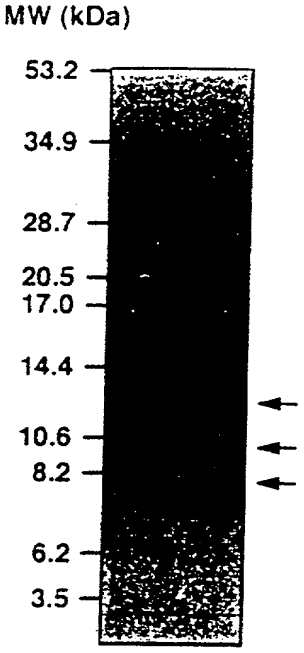


FIGURE 3

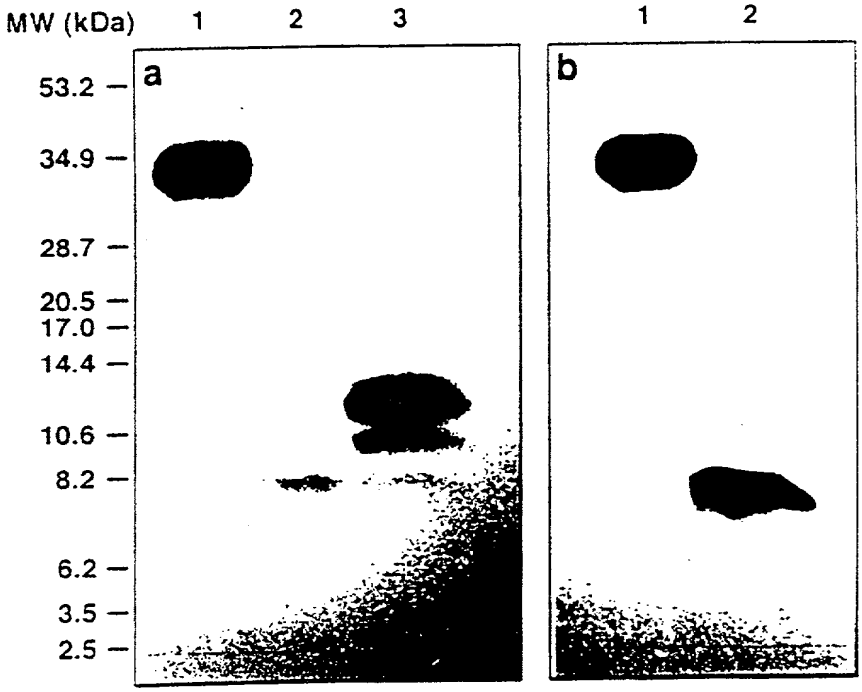


FIGURE 4

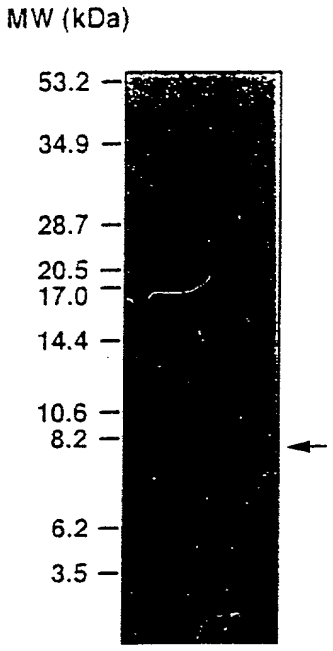


FIGURE 5

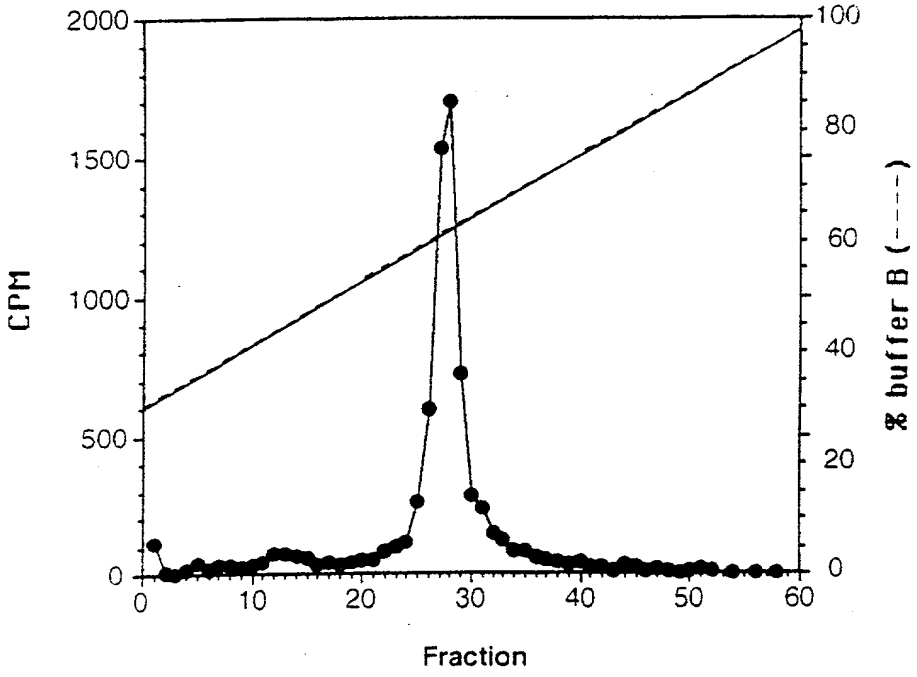

2000
1500
1000
500
0
CPM
0
10
20
30
40
50
60
Fraction
100
80
60
40
20
0
% buffer B (----)

FIGURE 6

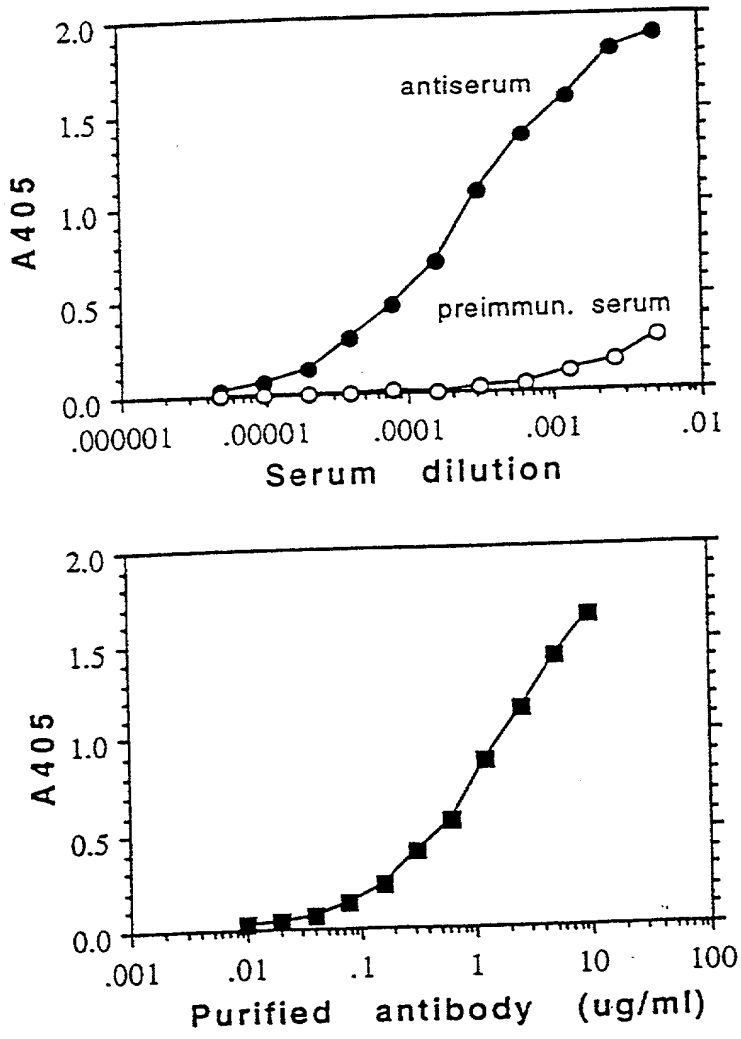

2.0
1.5
1.0
0.5
0.0
A405
antiserum
preimmun. serum
.000001
.00001
.0001
.001
.01
Serum dilution
2.0
1.5
1.0
0.5
0.0
A405
.001
.01
.1
1
10
100
Purified antibody (ug/ml)

FIGURE 7

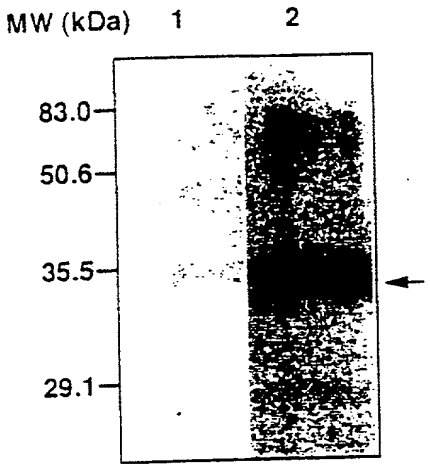


FIGURE 8

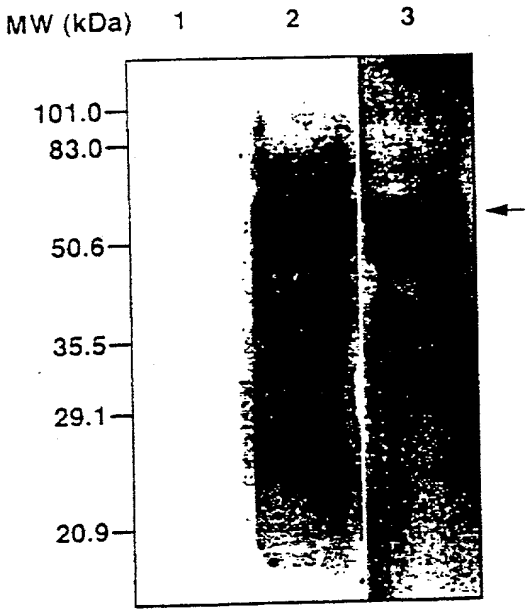


FIGURE 9

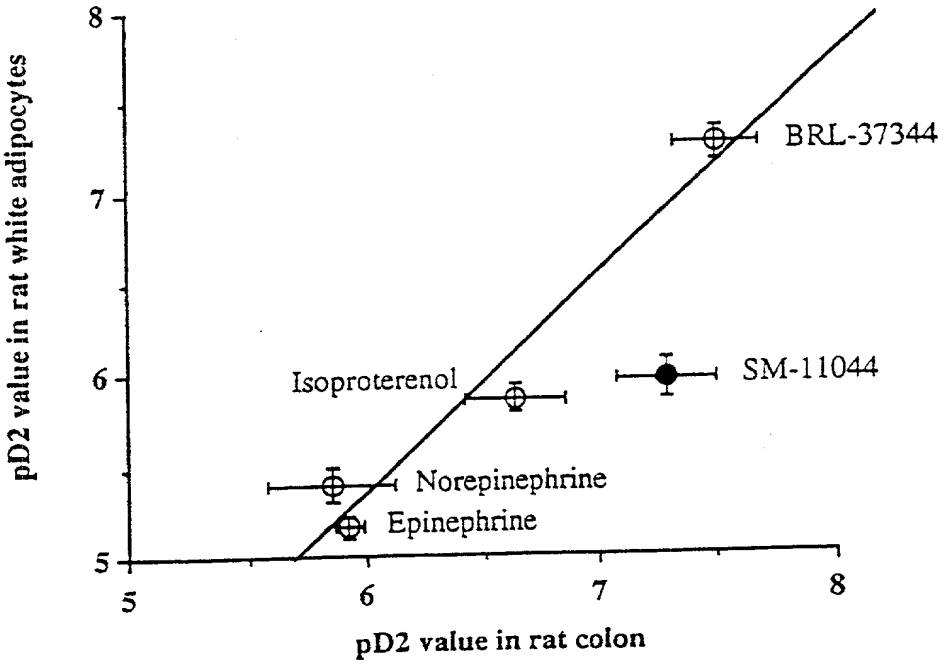

8
7
6
5
5
6
7
8
pD2 value in rat white adipocytes
pD2 value in rat colon
BRL-37344
SM-11044
Isoproterenol
Norepinephrine
Epinephrine

FIGURE 10

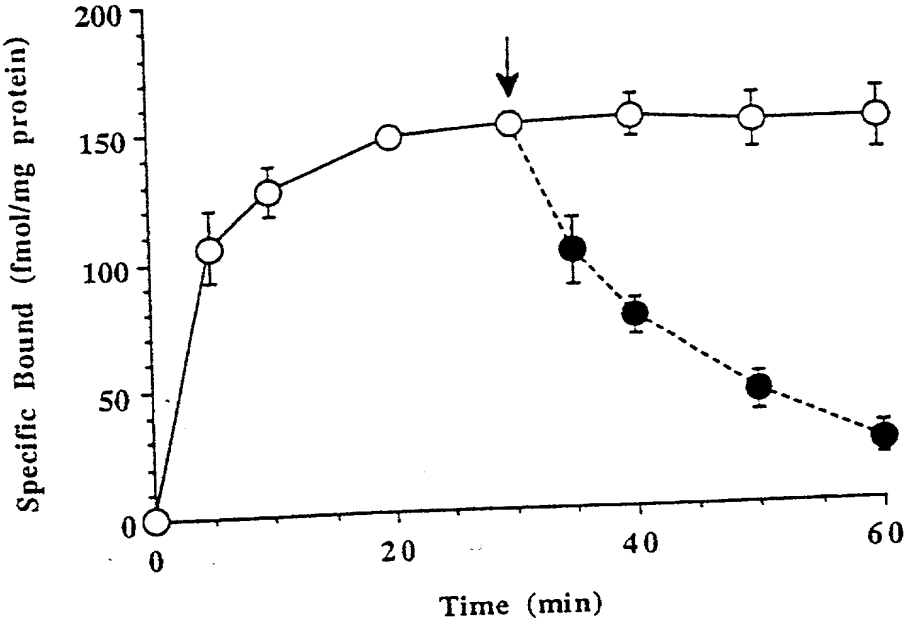

Specific Bound (fmol/mg protein)
200
150
100
50
0
0
20
40
60
Time (min)

FIGURE 11

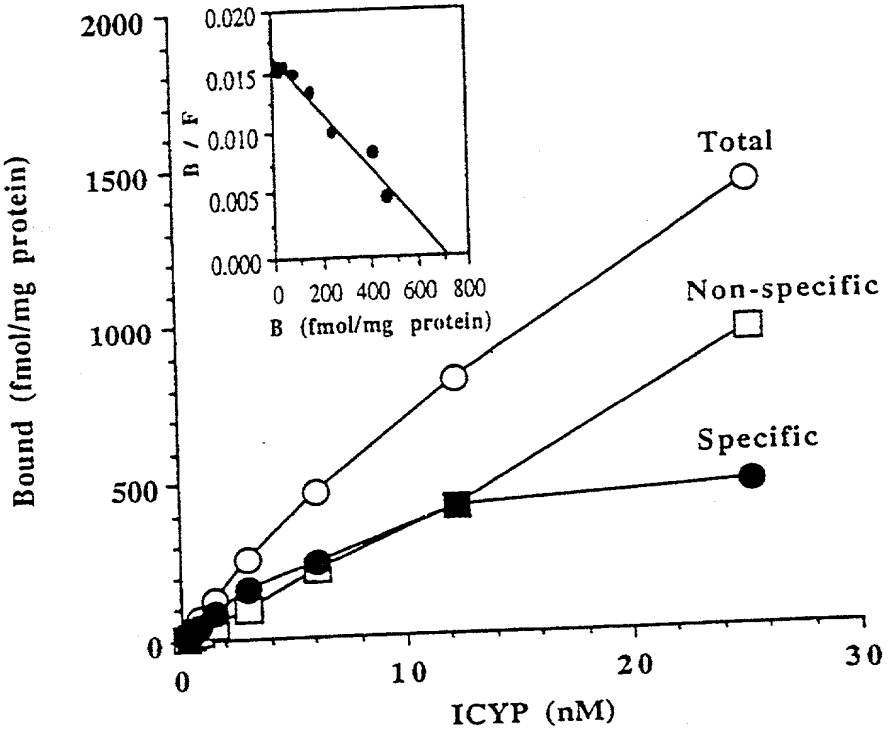

Bound (fmol/mg protein)
2000
1500
1000
500
0
0
10
20
30
ICYP (nM)
Total
Non-specific
Specific
B / F
0.020
0.015
0.010
0.005
0.000
0
200
400
600
800
B (fmol/mg protein)

FIGURE 12

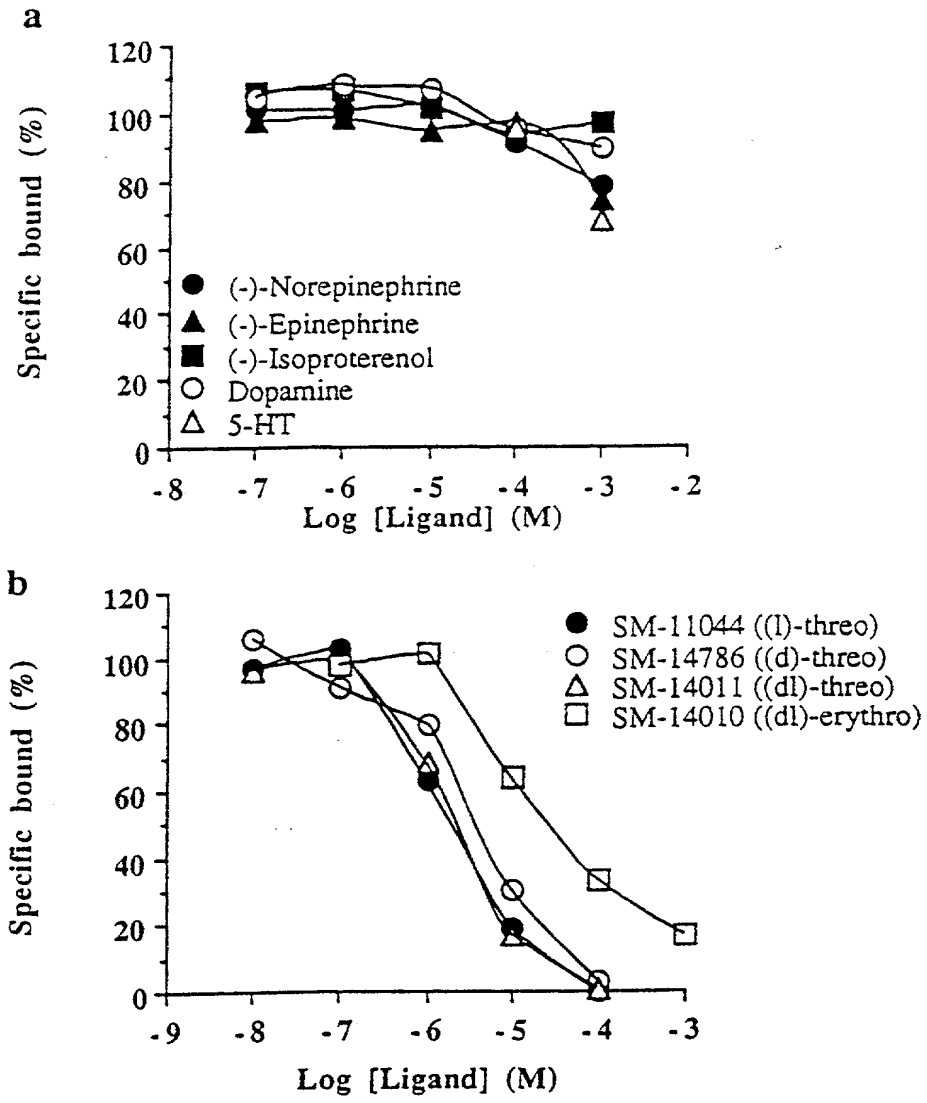

a
Specific bound (%)
120
100
80
60
40
20
0
(-)-Norepinephrine
(-)-Epinephrine
(-)-Isoproterenol
Dopamine
5-HT
-8
-7
-6
-5
-4
-3
-2
Log [Ligand] (M)
b
Specific bound (%)
120
100
80
60
40
20
0
SM-11044 ((l)-threo)
SM-14786 ((d)-threo)
SM-14011 ((dl)-threo)
SM-14010 ((dl)-erythro)
-9
-8
-7
-6
-5
-4
-3
Log [Ligand] (M)

FIGURE 13

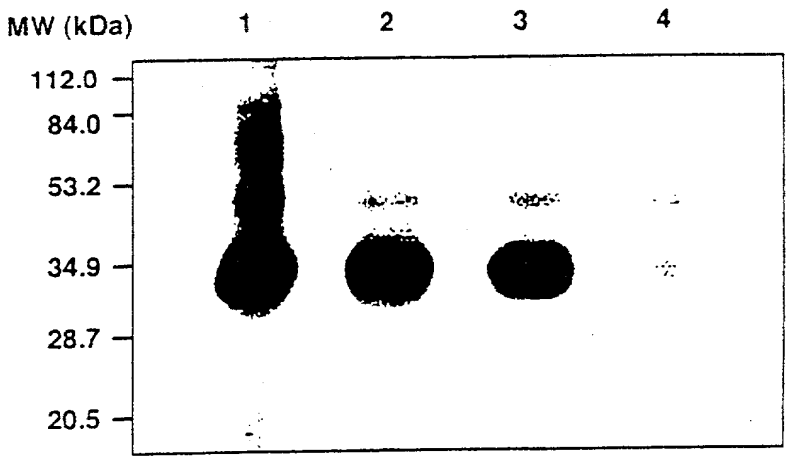


FIGURE 14

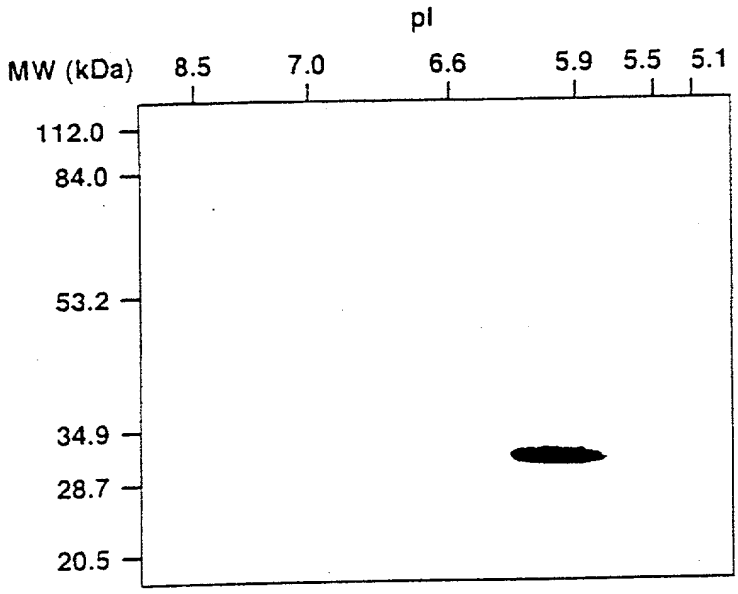


FIGURE 15

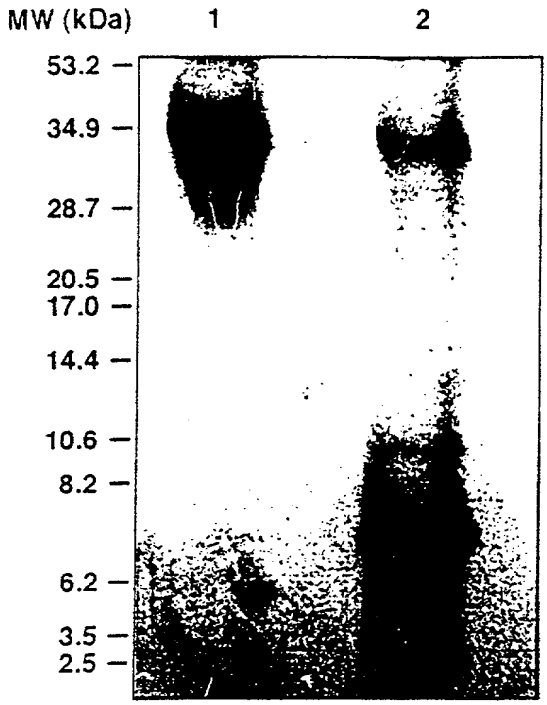


FIGURE 16

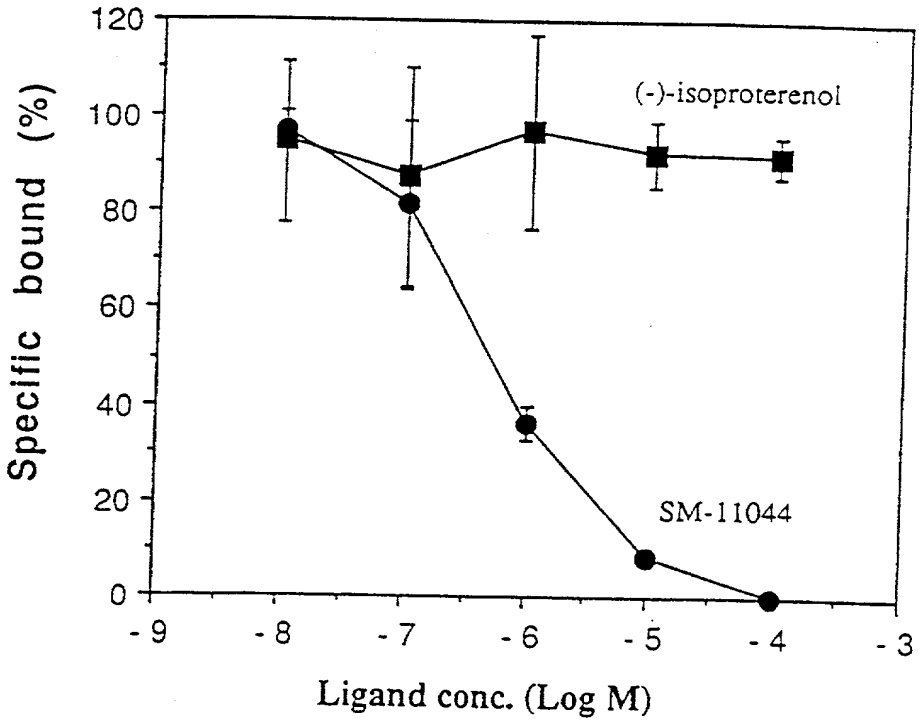
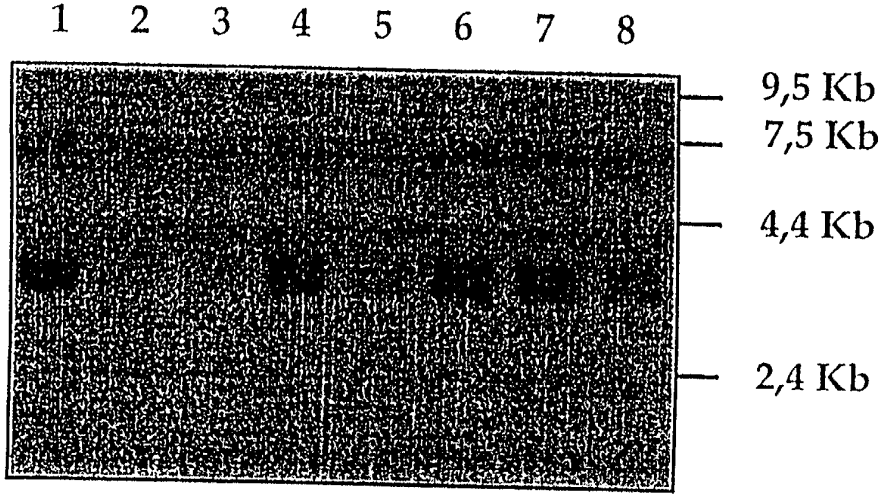

120
100
80
60
40
20
0
Specific bound (%)
(-)-isoproterenol
SM-11044
- 9
- 8
- 7
- 6
- 5
- 4
- 3
Ligand conc. (Log M)

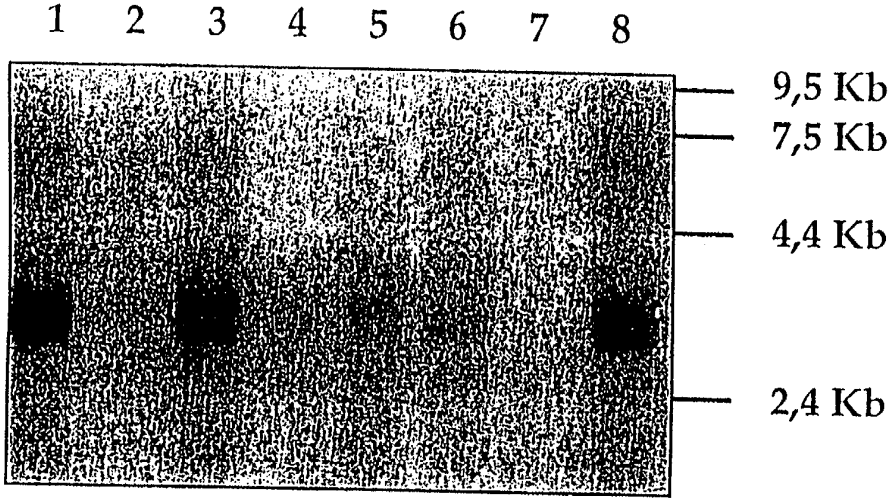
FIGURE 17



1 Skeletal muscle
2 Uterus (no endometrium)
3 Colon (no mucosa)
4 Small intestine
5 Bladder
6 Heart
7 Stomach
8 Prostate

FIGURE 18.A

668060 "h2613E60



Lane 1 : Heart
Lane 2 : Brain
Lane 3 : Placenta
Lane 4 : Lung
Lane 5 : Liver
Lane 6 : Skeletal muscle
Lane 7 : Kidney
Lane 8 : Pancreas

FIGURE 18.B

09/319724

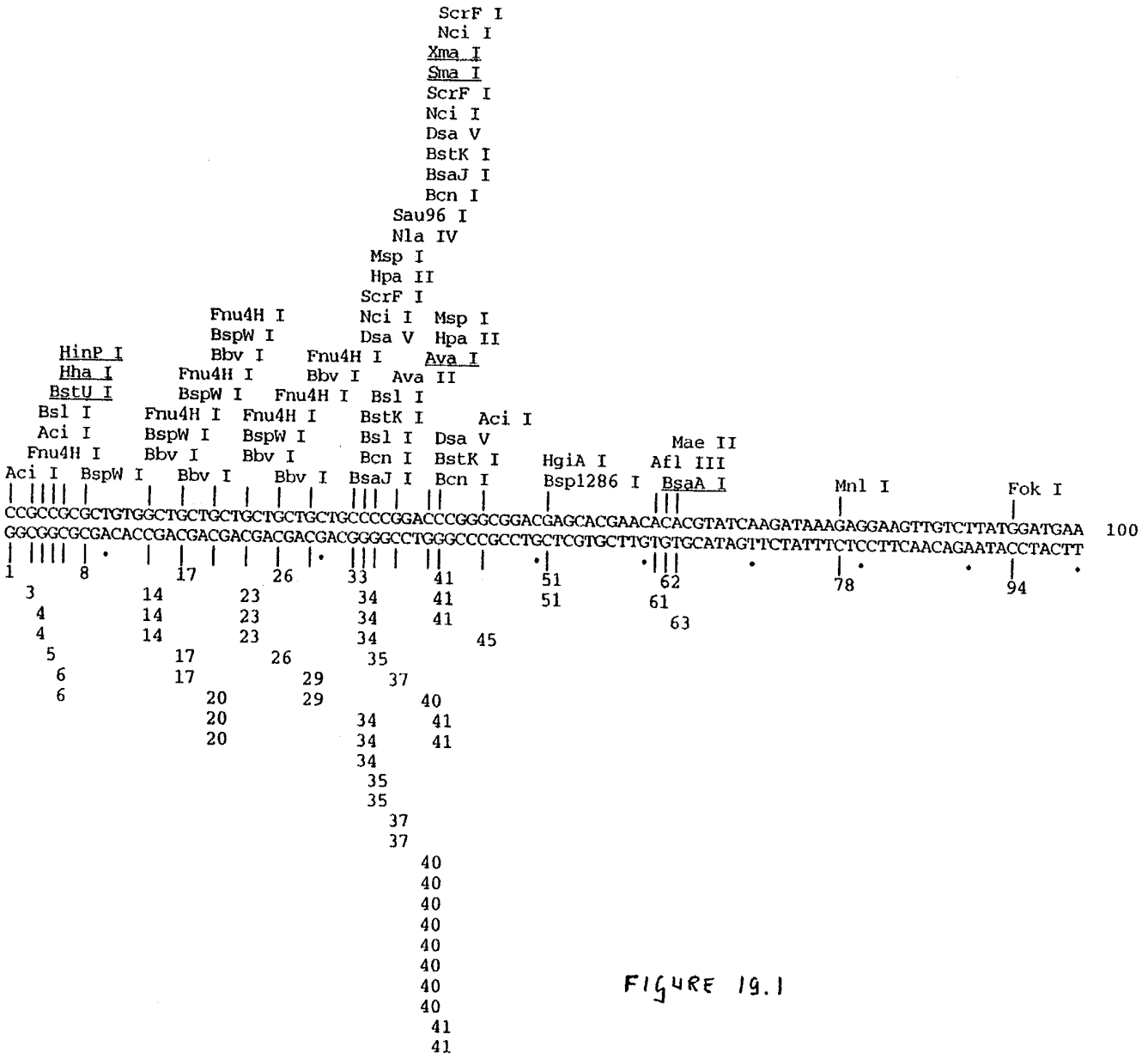

ScrF I
Nci I
Xma I
Sma I
ScrF I
Nci I
Dsa V
BstK I
BsaJ I
Bcn I
Sau96 I
Nla IV
Msp I
Hpa II
ScrF I
Fnu4H I
BspW I
Bbv I
Nci I
Msp I
Dsa V
Hpa II
HinP I
Hha I
BstU I
Fnu4H I
BspW I
Bbv I
Fnu4H I
Ava I
Bbv I
Ava II
Fnu4H I
BspW I
Fnu4H I
Bsl I
Bsl I
Aci I
Fnu4H I
Fnu4H I
BstK I
Aci I
BspW I
BspW I
Bsl I
Dsa V
Mae II
Bbv I
Bbv I
Bcn I
BstK I
HgiA I
Afl III
Aci I
BspW I
Bbv I
Bbv I
BsaJ I
Bcn I
Bsp1286 I
BsaA I
Mnl I
Fok I
CCGCCGCGCTGTGGCTGCTGCTGCTGCTGCCCCGGACCCGGGCGGACGAGCACGAACACACGTATCAAGATAAAGAGGAAGTTGTCTTATGGATGAA 100
GGCGGCGCGACACCGACGACGACGACGACGACGGGGCCTGGGCCCGCCTGCTCGTGCTTGTGTGCATAGTTCTATTTCTCCTTCAACAGAATACCTACTT
1 8 17 26 33 41 51 62 78 94
3 14 23 34 41 51 61
4 14 23 34 41 63
4 14 23 34 45
5 17 26 35
6 17 29 37
6 20 29 40
20 34 41
20 34 41
34
35
35
37
37
40
40
40
40
40
40
40
40
41
41

FIGURE 19.1

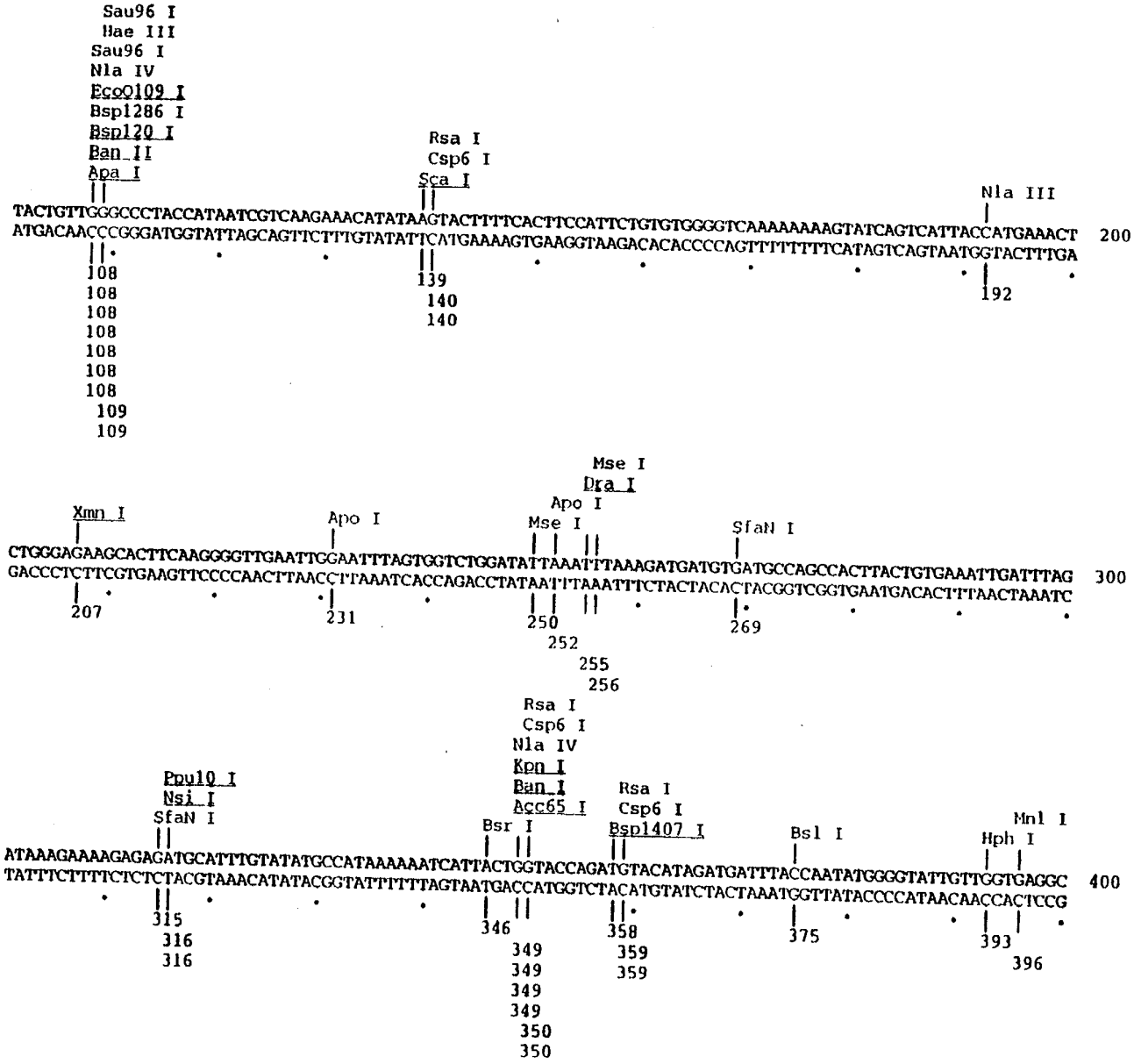

Sau96 I
Hae III
Sau96 I
Nla IV
EcoO109 I
Bsp1286 I
Bsp120 I
Ban II
Apa I
Rsa I
Csp6 I
Sca I
Nla III
TACTGTTGGGCCCTACCATAATCGTCAAGAAACATATAAGTACTTTTCACTTCCATTCTGTGTGGGGTCAAAAAAAAAGTATCAGTCATTACCATGAAACT 200
ATGACAACCCGGGATGGTATTAGCAGTTCTTTGTATATTCATGAAAAGTGAAGGTAAGACACACCCCAGTTTTTTTTCATAGTCAGTAATGGTACTTTGA
108
108
108
108
108
108
108
109
109
139
140
140
192
Mse I
Dra I
Apo I
Xmn I
Apo I
Mse I
SfaN I
CTGGGAGAAGCACTTCAAGGGGTTGAATTGGAATTTAGTGGTCTGGATATTAAATTTAAAGATGATGTGATGCCAGCCACTTACTGTGAAATTGATTTAG 300
GACCCTCTTCGTGAAGTTCCCCAACTTAACCTTAAATCACCAGACCTATAATTTAAATTTCTACTACACTACGGTCGGTGAATGACACTTTAACTAAATC
207
231
250
252
255
256
269
Rsa I
Csp6 I
Nla IV
Kpn I
Ban I
Acc65 I
Rsa I
Csp6 I
Bsp1407 I
Ppu10 I
Nsi I
SfaN I
Bsr I
Bsl I
Mnl I
Hph I
ATAAAGAAAAGAGAGATGCATTTGTATATGCCATAAAAAATCATTACTGGTACCAGATGTACATAGATGATTTACCAATATGGGGTATTGTTGGTGAGGC 400
TATTTCTTTTCTCTCTACGTAAACATATACGGTATTTTTTAGTAATGACCATGGTCTACATGTATCTACTAAATGGTTATACCCCATAACAACCACTCCG
315
316
316
346
349
349
349
349
350
350
358
359
359
375
393
396

FIGURE 19.2

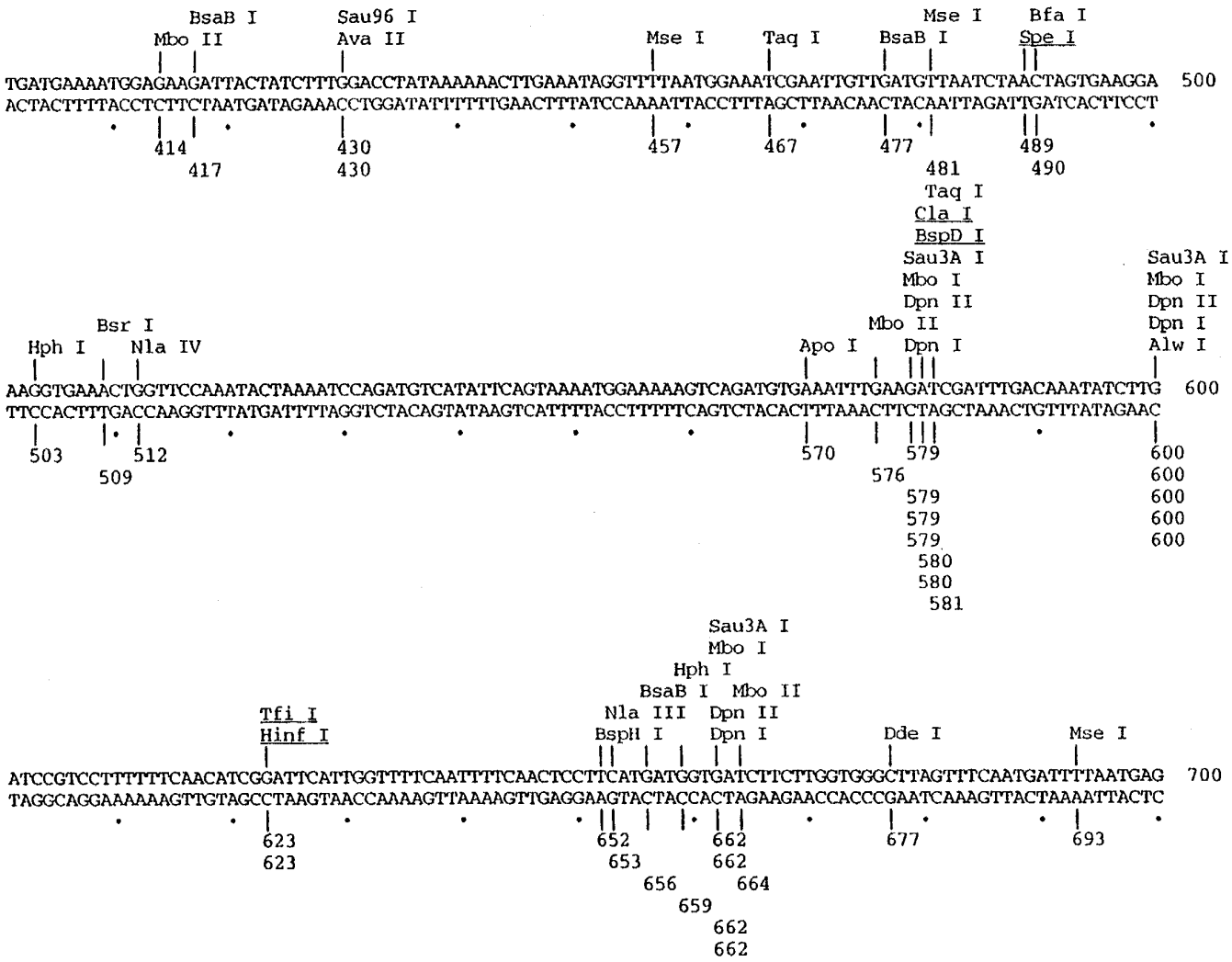
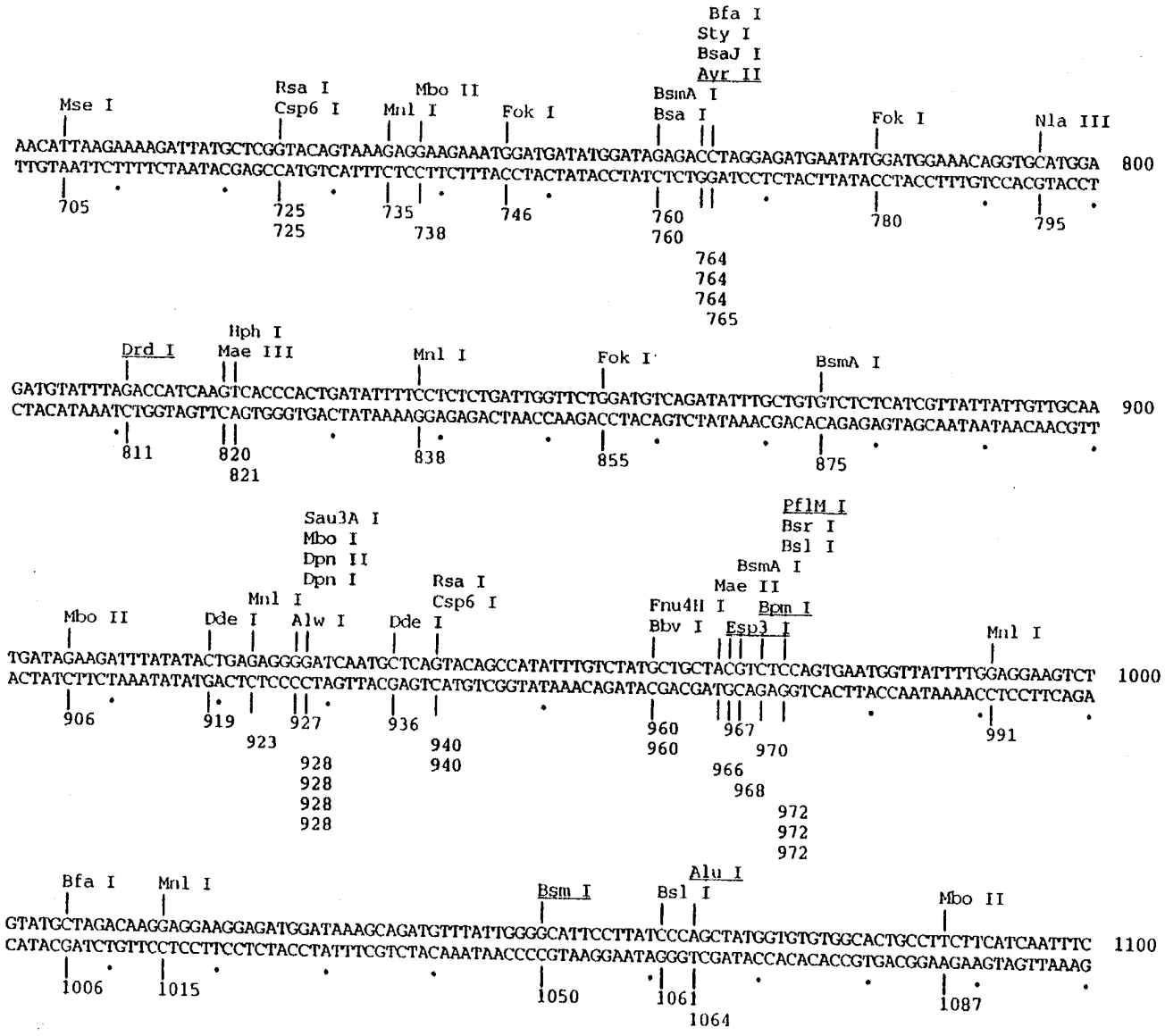

BsaB I
Mbo II
Sau96 I
Ava II
Mse I
Taq I
BsaB I
Mse I
Bfa I
Spe I
TGATGAAAATGGAGAAGATTACTATCTTTGGACCTATAAAAAACTTGAAATAGGTTTTAATGGAAATCGAATTGTTGATGTTAATCTAACTAGTGAAGGA 500
ACTACTTTTACCTCTTCTAATGATAGAAACCTGGATATTTTTTGAACTTTATCCAAAATTACCTTTAGCTTAACAACTACAATTAGATTGATCACTTCCT
414
417
430
430
457
467
477
481
489
490
Taq I
Cla I
BspD I
Sau3A I
Mbo I
Dpn II
Sau3A I
Mbo I
Dpn II
Dpn I
Alw I
Bsr I
Hph I
Nla IV
Mbo II
Apo I
Dpn I
AAGGTGAAACTGGTTCCAAATACTAAAATCCAGATGTCATATTCAGTAAAATGGAAAAAGTCAGATGTGAAATTTGAAGATCGATTTGACAAATATCTTG 600
TTCCACTTTGACCAAGGTTTATGATTTTAGGTCTACAGTATAAGTCATTTTACCTTTTTCAGTCTACACTTTAAACTTCTAGCTAAACTGTTTATAGAAC
503
509
512
570
576
579
579
579
579
580
580
581
600
600
600
600
600
Sau3A I
Mbo I
Hph I
BsaB I
Mbo II
Tfi I
Hinf I
Nla III
Dpn II
BspH I
Dpn I
Dde I
Mse I
ATCCGTCCTTTTTTCAACATCGGATTCATTGGTTTTCAATTTTCAACTCCTTCATGATGGTGATCTTCTTGGTGGGCTTAGTTTCAATGATTTTAATGAG 700
TAGGCAGGAAAAAAGTTGTAGCCTAAGTAACCAAAAGTTAAAAGTTGAGGAAGTACTACCACTAGAAGAACCACCCGAATCAAAGTTACTAAAATTACTC
623
623
652
653
656
659
662
662
662
664
677
693

FIGURE 19.3


Bfa I
Sty I
BsaJ I
Avr II
Rsa I
Csp6 I
Mbo II
Mnl I
Fok I
BsmA I
Bsa I
Mse I
Fok I
Nla III
AACATTAAGAAAAGATTATGCTCGGTACAGTAAAGAGGAAGAAATGGATGATATGGATAGAGACCTAGGAGATGAATATGGATGGAAACAGGTGCATGGA 800
TTGTAATTCTTTTCTAATACGAGCCATGTCATTTCTCCTTCTTTACCTACTATACCTATCTCTGGATCCTCTACTTATACCTACCTTTGTCCACGTACCT
705
725
725
735
738
746
760
760
764
764
764
765
780
795
Drd I
Hph I
Mae III
Mnl I
Fok I
BsmA I
GATGTATTTAGACCATCAAGTCACCCACTGATATTTTCCTCTCTGATTGGTTCTGGATGTCAGATATTTGCTGTGTCTCTCATCGTTATTATTGTTGCAA 900
CTACATAAATCTGGTAGTTCAGTGGGTGACTATAAAAGGAGAGACTAACCAAGACCTACAGTCTATAAACGACACAGAGAGTAGCAATAATAACAACGTT
811
820
821
838
855
875
Sau3A I
Mbo I
Dpn II
Dpn I
PflM I
Bsr I
Bsl I
BsmA I
Mae II
Mnl I
Rsa I
Csp6 I
Fnu4H I
Bpm I
Mbo II
Dde I
Alw I
Dde I
Bbv I
Esp3 I
Mnl I
TGATAGAAGATTTATATACTGAGAGGGGATCAATGCTCAGTACAGCCATATTTGTCTATGCTGCTACGTCTCCAGTGAATGGTTATTTTGGAGGAAGTCT 1000
ACTATCTTCTAAATATATGACTCTCCCCTAGTTACGAGTCATGTCGGTATAAACAGATACGACGATGCAGAGGTCACTTACCAATAAAACCTCCTTCAGA
906
919
923
927
928
928
928
928
936
940
940
960
960
966
967
968
970
972
972
972
991
Bfa I
Mnl I
Alu I
Bsm I
Bsl I
Mbo II
GTATGCTAGACAAGGAGGAAGGAGATGGATAAAGCAGATGTTTATTGGGGCATTCCTTATCCCAGCTATGGTGTGTGGCACTGCCTTCTTCATCAATTTC 1100
CATACGATCTGTTCCTCCTTCCTCTACCTATTTCGTCTACAAATAACCCCGTAAGGAATAGGGTCGATACCACACACCGTGACGGAAGAAGTAGTTAAAG
1006
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1050
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1064
1087

FIGURE 19.4

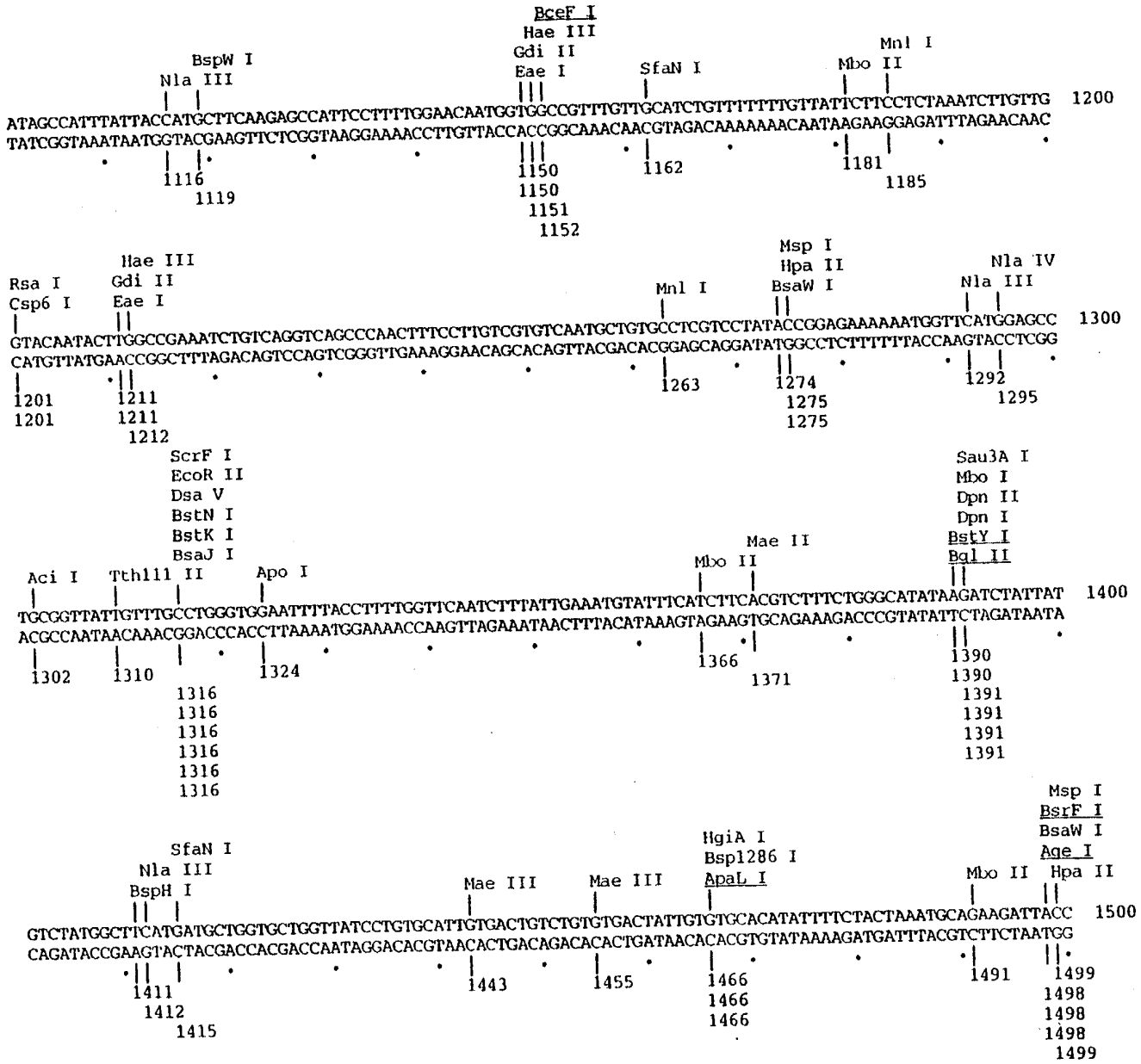
BceF I
Hae III
Gdi II
Eae I
BspW I
Nla III
SfaN I
Mnl I
Mbo II
ATAGCCATTTATTACCATGCTTCAAGAGCCATTCCTTTTGGAACAATGGTGGCCGTTTGTTGCATCTGTTTTTTTGTTATTCTTCCTCTAAATCTTGTTG 1200
TATCGGTAAATAATGGTACGAAGTTCTCGGTAAGGAAAACCTTGTTACCACCGGCAAACAACGTAGACAAAAAAACAATAAGAAGGAGATTTAGAACAAC
1116
1119
1150
1150
1151
1152
1162
1181
1185
Rsa I
Csp6 I
Hae III
Gdi II
Eae I
Mnl I
Msp I
Hpa II
BsaW I
Nla IV
Nla III
GTACAATACTTGGCCGAAATCTGTCAGGTCAGCCCAACTTTCCTTGTCGTGTCAATGCTGTGCCTCGTCCTATACCGGAGAAAAAATGGTTCATGGAGCC 1300
CATGTTATGAACCGGCTTTAGACAGTCCAGTCGGGTTGAAAGGAACAGCACAGTTACGACACGGAGCAGGATATGGCCTCTTTTTTACCAAGTACCTCGG
1201
1201
1211
1211
1212
1263
1274
1275
1275
1292
1295
ScrF I
EcoR II
Dsa V
BstN I
BstK I
BsaJ I
Sau3A I
Mbo I
Dpn II
Dpn I
BstY I
Bgl II
Aci I
Tth111 II
Apo I
Mae II
Mbo II
TGCGGTTATTGTTTGCCTGGGTGGAATTTTACCTTTTGGTTCAATCTTTATTGAAATGTATTTCATCTTCACGTCTTTCTGGGCATATAAGATCTATTAT 1400
ACGCCAATAACAAACGGACCCACCTTAAAATGGAAAACCAAGTTAGAAATAACTTTACATAAAGTAGAAGTGCAGAAAGACCCGTATATTCTAGATAATA
1302
1310
1316
1316
1316
1316
1316
1316
1324
1366
1371
1390
1390
1391
1391
1391
1391
SfaN I
Nla III
BspH I
Mae III
Mae III
HgiA I
Bsp1286 I
ApaL I
Msp I
BsrF I
BsaW I
Age I
Mbo II
Hpa II
GTCTATGGCTTCATGATGCTGGTGCTGGTTATCCTGTGCATTGTGACTGTCTGTGTGACTATTGTGTGCACATATTTTCTACTAAATGCAGAAGATTACC 1500
CAGATACCGAAGTACTACGACCACGACCAATAGGACACGTAACACTGACAGACACACTGATAACACACGTGTATAAAAGATGATTTACGTCTTCTAATGG
1411
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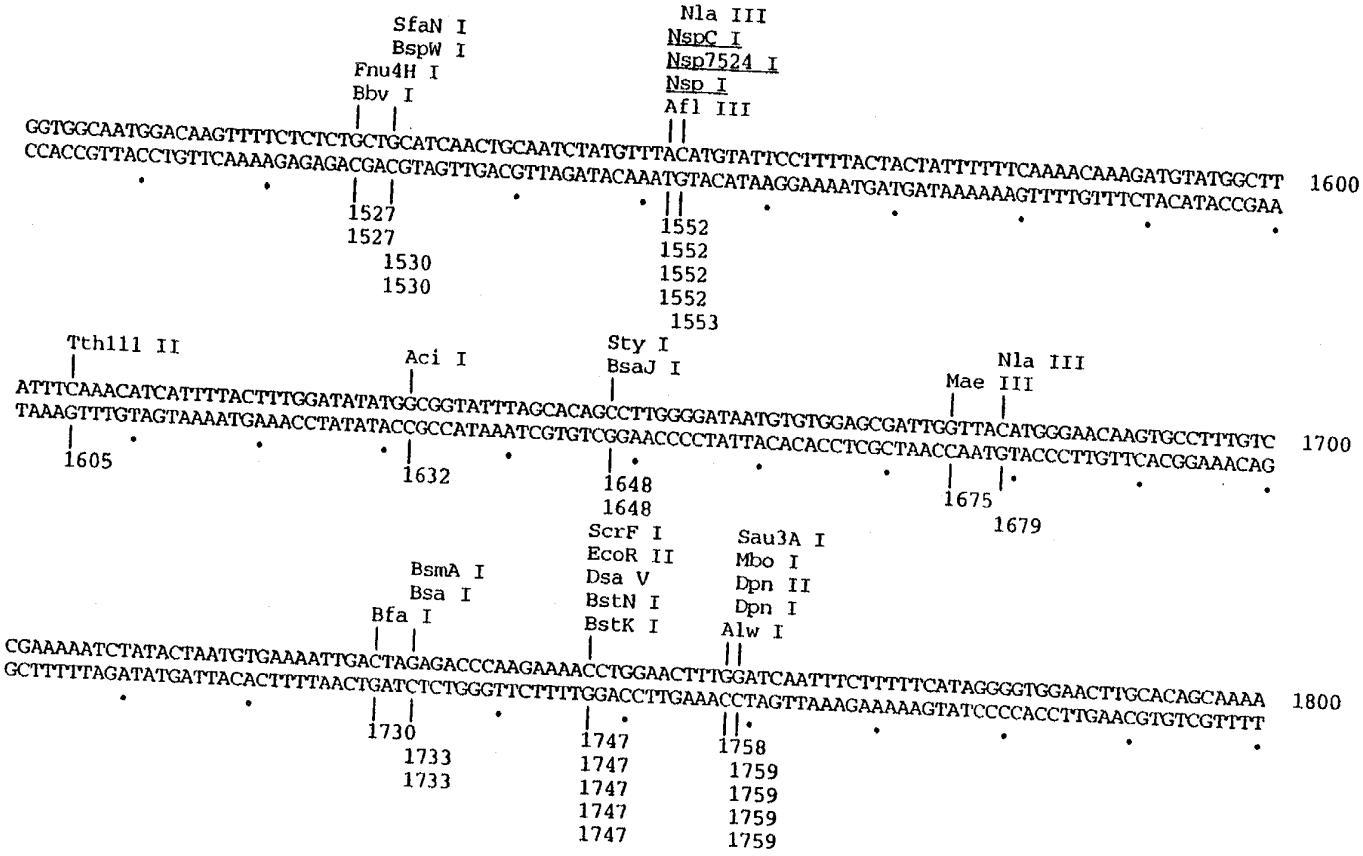
FIGURE 19.5


SfaN I
BspW I
Fnu4H I
Bbv I
Nla III
NspC I
Nsp7524 I
Nsp I
Afl III
GGTGGCAATGGACAAGTTTTCTCTCTGCTGCATCAACTGCAATCTATGTTTACATGTATTCCTTTTACTACTATTTTTTCAAAACAAAGATGTATGGCTT 1600
CCACCGTTACCTGTTCAAAAGAGAGACGACGTAGTTGACGTTAGATACAAATGTACATAAGGAAAATGATGATAAAAAAGTTTTGTTTCTACATACCGAA
1527
1527
1530
1530
1552
1552
1552
1552
1553
Tth111 II
Aci I
Sty I
BsaJ I
Nla III
Mae III
ATTTCAAACATCATTTTACTTTGGATATATGGCGGTATTTAGCACAGCCTTGGGGATAATGTGTGGAGCGATTGGTTACATGGGAACAAGTGCCTTTGTC 1700
TAAAGTTTGTAGTAAAATGAAACCTATATACCGCCATAAATCGTGTCGGAACCCCTATTACACACCTCGCTAACCAATGTACCCTTGTTCACGGAAACAG
1605
1632
1648
1648
1675
1679
ScrF I
EcoR II
Dsa V
BstN I
BstK I
Sau3A I
Mbo I
Dpn II
Dpn I
Alw I
BsmA I
Bsa I
Bfa I
CGAAAAATCTATACTAATGTGAAAATTGACTAGAGACCCAAGAAAACCTGGAACTTTGGATCAATTTCTTTTTCATAGGGGTGGAACTTGCACAGCAAAA 1800
GCTTTTTAGATATGATTACACTTTTAACTGATCTCTGGGTTCTTTTGGACCTTGAAACCTAGTTAAAGAAAAAGTATCCCCACCTTGAACGTGTCGTTTT
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1759

FIGURE 19.6

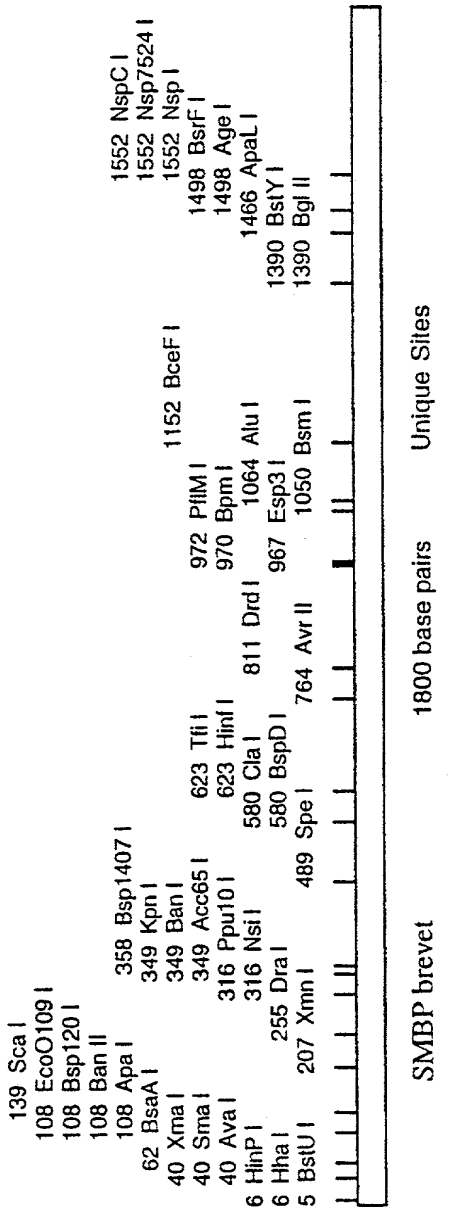

139 Sca I
108 EcoO109 I
108 Bsp120 I
108 Ban II
108 Apa I
62 BsaA I
40 Xma I
40 Sma I
40 Ava I
6 HinP I
6 Hha I
5 BstU I
358 Bsp1407 I
349 Kpn I
349 Ban I
349 Acc65 I
316 Ppu10 I
316 Nsi I
255 Dra I
207 Xmn I
623 Tfi I
623 Hinf I
580 Cla I
580 BspD I
489 Spe I
811 Drd I
764 Avr II
1152 BceF I
972 PflM I
970 Bpm I
1064 Alu I
967 Esp3 I
1050 Bsm I
1552 NspC I
1552 Nsp7524 I
1552 Nsp I
1498 BsrF I
1498 Age I
1466 ApaL I
1390 BstY I
1390 Bgl II
SMBP brevet
1800 base pairs
Unique Sites

FIGURE 20

Enzyme	Site	<--	Pos.	-->
BstU I	cg/cg	4	5	1796
Hha I	gcg/c	5	6	1795
HinP I	g/cgc	5	6	1795
Ava I	c/ycgrg	39	40	1761
Sma I	ccc/ggg	39	40	1761
Xma I	c/ccggg	39	40	1761
BsaA I	yac/gtr	61	62	1739
Apa I	gggcc/c	107	108	1693
Ban II	grgcy/c	107	108	1693
Bsp120 I	g/ggccc	107	108	1693
EcoO109 I	rg/gnccy	107	108	1693
Sca I	agt/act	138	139	1662
Xmn I	gaann/nnttc	206	207	1594
Dra I	ttt/aaa	254	255	1546
Nsi I	atgca/t	315	316	1485
Ppu10 I	a/tgcat	315	316	1485
Acc65 I	g/gtacc	348	349	1452
Ban I	g/gyrcc	348	349	1452
Kpn I	ggtac/c	348	349	1452
Bsp1407 I	t/gtaca	357	358	1443
Spe I	a/ctagt	488	489	1312
BspD I	at/cgat	579	580	1221
Cla I	at/cgat	579	580	1221
Hinf I	g/antc	622	623	1178
Tfi I	g/awtc	622	623	1178
Avr II	c/ctagg	763	764	1037
Drd I	gacnnnn/nngtc	810	811	990
Esp3 I	cgtctc 1/5	966	967	834
Bpm I	ctggag 16/14	969	970	831
PflM I	ccannnn/ntgg	971	972	829
Bsm I	gaatgc 1/-1	1049	1050	751
Alu I	ag/ct	1063	1064	737
BceF I	acggc 12/13	1151	1152	649
Bgl II	a/gatct	1389	1390	411
BstY I	r/gatcy	1389	1390	411
ApaL I	g/tgcac	1465	1466	335
Age I	a/ccggt	1497	1498	303
BsrF I	r/ccggy	1497	1498	303
Nsp I	rcatg/y	1551	1552	249
Nsp7524 I	r/catgy	1551	1552	249
NspC I	rcatg/y	1551	1552	249

FIGURE 21

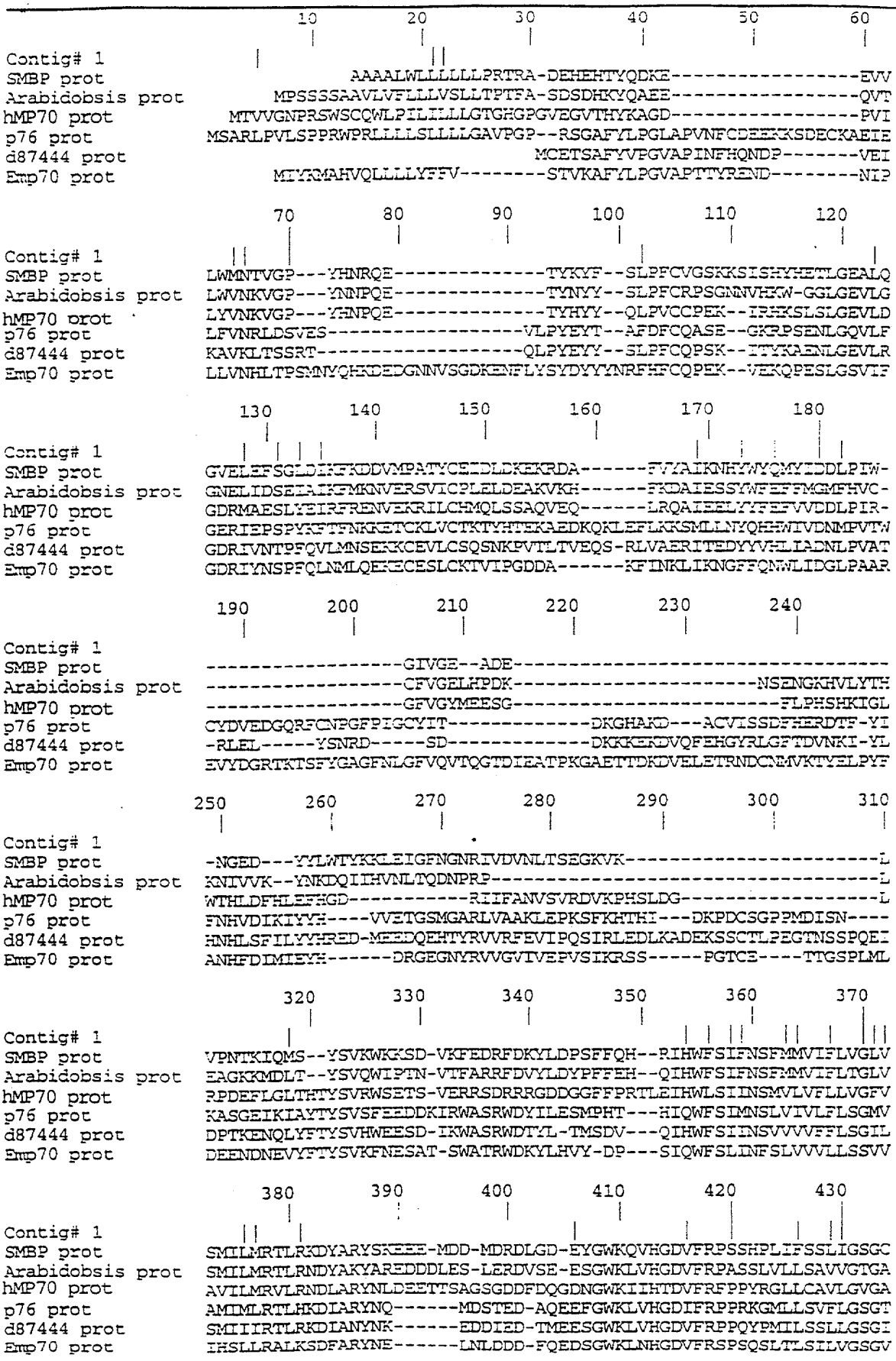

10 20 30 40 50 60
Contig# 1
SMBP prot AAAALWLLLLLLPRTRA-DEHEHTYQDKE----------EVV
Arabidobsis prot MPSSSSAAVLVFLLLVSLLTPTFA-SDSDHKYQAEE----------QVT
hMP70 prot MTVVGNPRSWSCQWLPILILLLGTGHGPGVEGVTHYKAGD----------PVI
p76 prot MSARLPVLSPPRWPRLLLLSLLLLGAVPGP--RSGAFYLPGLAPVNFCDEEKKSDECKAEIE
d87444 prot MCETSAFYVPGVAPINFHQNDP-------VEI
Emp70 prot MIYKMAHVQLLLLYFFV--------STVKAFYLPGVAPTTYRENDP--------NIP
70 80 90 100 110 120
Contig# 1
SMBP prot LWMNTVGP---YHNRQE----------TYKYF--SLPFCVGSKKSISHYHETLGEALQ
Arabidobsis prot LWVNKVGP---YNNPQE----------TYNYY--SLPFCRPSGNNVHKW-GGLGEVLG
hMP70 prot LYVNKVGP---YHNPQE----------TYHYY--QLPVCCPEK--IRHKSLSLGEVLD
p76 prot LFVNRLDSVES----------VLPYEYT--AFDFCQASE--GKRPSENLGQVLF
d87444 prot KAVKLTSSRT----------QLPYEYY--SLPFCQPSK--ITYKAENLGEVLR
Emp70 prot LLVNHLTPSMNYQHKDEDGNNVSGDKENFLYSYDYYYNRFHFCQPEK--VEKQPESLGSVIF
130 140 150 160 170 180
Contig# 1
SMBP prot GVELEFSGLDIKFKDDVMPATYCEIDLDKEKRDA------FVYAIKNHYWYQMYIDDLPIW-
Arabidobsis prot GNELIDSEIAIKFMKNVERSVICPLELDEAKVKH------FKDAIESSYWFEFFMGMFHVC-
hMP70 prot GDRMAESLYEIRFRENVEKRILCHMQLSSAQVEQ------LRQAIEELYTFEFVVDDLPIR-
p76 prot GERIEPSPYKFTFNKKETCKLVCTKTYHTEKAEDKQKLEFLAKSMLLNYQHHWIVDNMPVTW
d87444 prot GDRIVNTPFQVLMNSEKKCEVLCSQSNKPVTLTVEQS-RLVAERITEDYYVHLIADNLPVAT
Emp70 prot GDRIYNSPFQLNMLQEKECESLCKTVIPGDDA------KFINKLIKNGFFQNWLIDGLPAAR
190 200 210 220 230 240
Contig# 1
SMBP prot ----------GIVGE--ADE----------
Arabidobsis prot ----------CFVGELHPDK----------NSENGKHVLYTH
hMP70 prot ----------GFVGYMEESG----------FLPHSHKIGL
p76 prot CYDVEDGQRFCNPGFPIGCYIT----------DKGHAKD---ACVISSDFHERDTF-YI
d87444 prot -RLEL-----YSNRD-----SD----------DKKKENDVQFEHGYRLGFTDVNKI-YL
Emp70 prot EVYDGRTKTSFYGAGFNLGFVQVTQGTDIEATPKGAETTDKDVELETRNDCNMVKTYELPYF
250 260 270 280 290 300 310
Contig# 1
SMBP prot -NGED---YYLWTYKKLEIGFNGNRIVDVNLTSEGKVK----------L
Arabidobsis prot KNIVVK--YNKDQIIHVNLTQDNPRP----------L
hMP70 prot WTHLDFHLEFHGD----------RIIFANVSVRDVKPHSLDG----------L
p76 prot FNHVDIKIYYH----VVETGSMGARLVAAKLEPKSFKHTHI---DKPDCSGPPMDISN----
d87444 prot HNHLSFILYYHRED-MEEDQEHTYRVVRFEVIPQSIRLEDLKADEKSSCTLPEGTNSSPQEI
Emp70 prot ANHFDIMIEYH------DRGEGNYRVVGVIVEPVSIKRSS-----PGTCE----TTGSPLML
320 330 340 350 360 370
Contig# 1
SMBP prot VPNTKIQMS--YSVKWKKSD-VKFEDRFDKYLDPSFFQH--RIHWFSIFNSFMMVIFLVGLV
Arabidobsis prot EAGKKMDLT--YSVQWIPTN-VTFARRFDVYLDYPFFEH--QIHWFSIFNSFMMVIFLTGLV
hMP70 prot RPDEFLGLTHTYSVRWSETS-VERRSDRRRGDDGGFFPRTLEIHWLSIINSMVLVFLLVGFV
p76 prot KASGEIKIAYTYSVSFEEDDKIRWASRWDYILESMPHT---HIQWFSIMNSLVIVLFLSGMV
d87444 prot DPTKENQLYFTYSVHWEESD-IKWASRWDTYL-TMSDV---QIHWFSIINSVVVVFFLSGIL
Emp70 prot DEENDNEVYFTYSVKFNESAT-SWATRWDKYLHVY-DP---SIQWFSLINFSLVVVLLSSVV
380 390 400 410 420 430
Contig# 1
SMBP prot SMILMRTLRKDYARYSKEEE-MDD-MDRDLGD-EYGWKQVHGDVFRPSSHPLIFSSLIGSGC
Arabidobsis prot SMILMRTLRNDYAKYAREDDDLES-LERDVSE-ESGWKLVHGDVFRPASSLVLLSAVVGTGA
hMP70 prot AVILMRVLRNDLARYNLDEETTSAGSGDDFDQGDNGWKIIHTDVFRFPPYRGLLCAVLGVGA
p76 prot AMIMLRTLHKDIARYNQ------MDSTED-AQEEFGWKLVHGDIFRPPRKGMLLSVFLGSGT
d87444 prot SMIIIRTLRKDIANYNK------EDDIED-TMEESGWKLVHGDVFRPPQYPMILSSLLGSGI
Emp70 prot IHSLLRALKSDFARYNE------LNLDDD-FQEDSGWKLNHGDVFRSPSQSLTLSILVGSGV

FIGURE 22.1

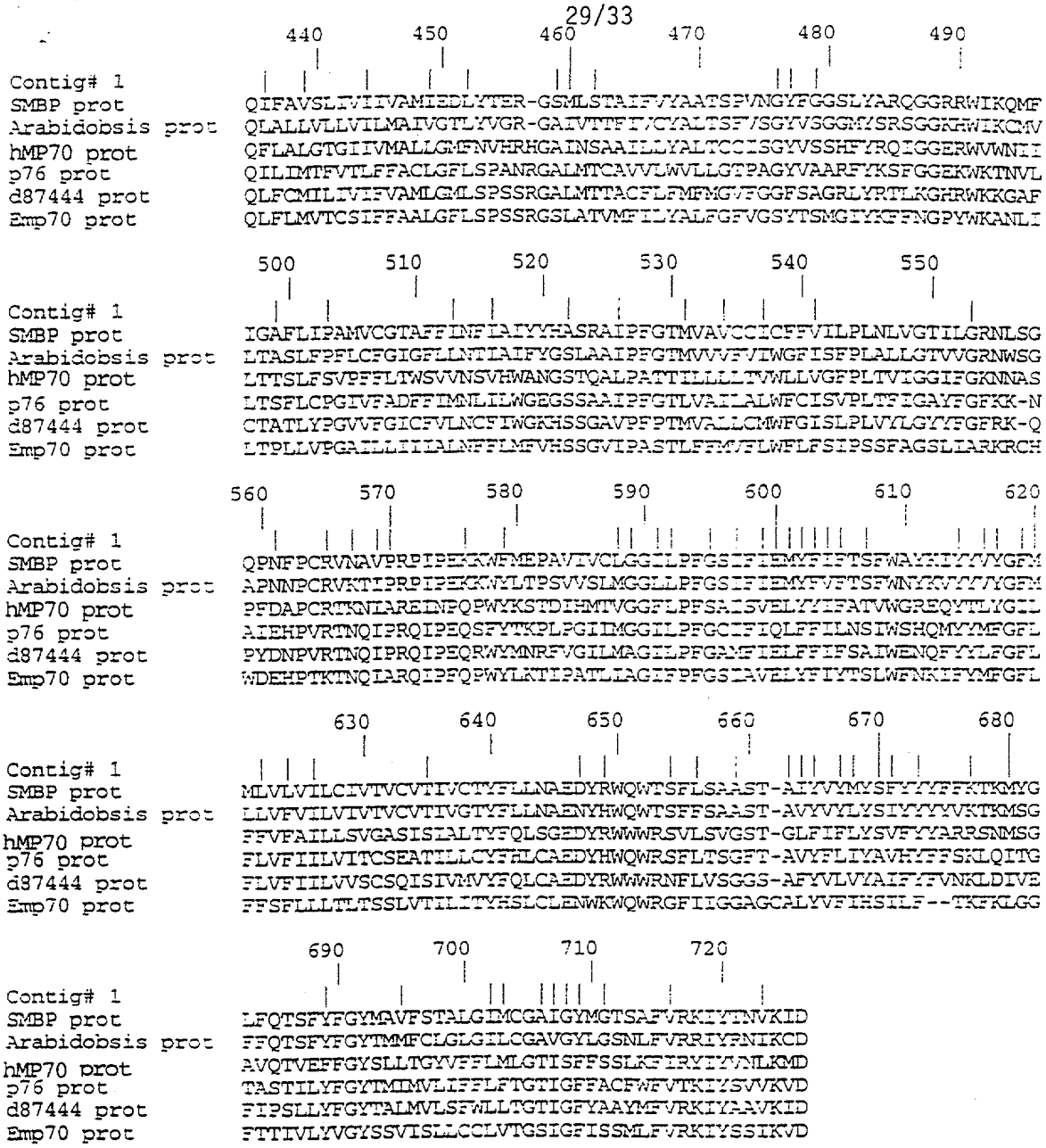
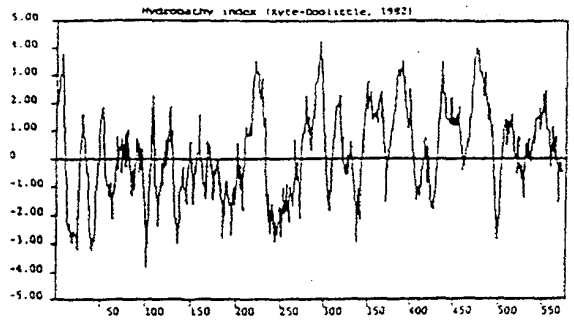
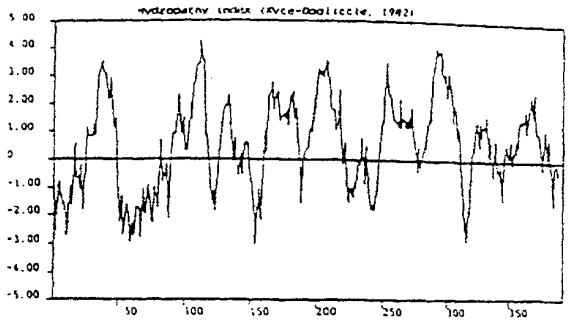

440 450 460 470 480 490
Contig# 1
SMBP prot QIFAVSLIVIIVAMIEDLYTER-GSMLSTAIFVYAATSPVNGYFGGSLYARQGGRRWIKQMF
Arabidobsis prot QLALLVLLVILMAIVGTLYVGR-GAIVTTFIYCYALTSFYSGYVSGGMYSRSGGKRWIKCMV
hMP70 prot QFLALGTGIIVMALLGMFNVHRHGAINSAAILLYALTCCISGYVSSHFYRQIGGERWVWNII
p76 prot QILIMTFVTLFFACLGFLSPANRGALMTCAVVLWVLLGTPAGYVAARFYKSFGGEKWKTNVL
d87444 prot QLFCMILIVIFVAMLGMLSPSSRGALMTTACFLFMFMGVFGGFSAGRLYRTLKGHRWKKGAF
Emp70 prot QLFLMVTCSIFFAALGFLSPSSRGSLATVMFILYALFGFVGSYTSMGIYKFFNGPYWKANLI
500 510 520 530 540 550
Contig# 1
SMBP prot IGAFLIPAMVCGTAFFINFIAIYYHASRAIPFGTMVAVCCICFFVILPLNLVGTILGRNLSG
Arabidobsis prot LTASLFPFLCFGIGFLLNTIAIFYGSLAAIPFGTMVVVFVIWGFISFPLALLGTVVGRNWSG
hMP70 prot LTTSLFSVPFFLTWSVVNSVHWANGSTQALPATTILLLLTVWLLVGFPLTVIGGIFGKNNAS
p76 prot LTSFLCPGIVFADFFIMNLILWGEGSSAAIPFGTLVAILALWFCISVPLTFIGAYFGFKK-N
d87444 prot CTATLYPGVVFGICFVLNCFIWGKHSSGAVPFPTMVALLCMWFGISLPLVYLGYYFGFRK-Q
Emp70 prot LTPLLVPGAILLIIIALNFFLMFVHSSGVIPASTLFFMVFLWFLFSIPSSFAGSLIARKRCH
560 570 580 590 600 610 620
Contig# 1
SMBP prot QPNFPCRVNAVPRPIPEKKWFMEPAVIVCLGGILPFGSIFIEMYFIFTSFWAYHIYVVYGFM
Arabidobsis prot APNNPCRVKTIPRPIPEKGNYLTPSVVSLMGGLLPFGSIFIEMYFVFTSFWNYKVYVYYGFM
hMP70 prot PFDAPCRTKNIAREINPQPWYKSTDIHMTVGGFLPFSAISVELYYIFATVWGREQYTLYGIL
p76 prot AIEHPVRTNQIPRQIPEQSFYTKPLPGIIMGGILPFGCIFIQLFFILNSIWSHQMYYMFGFL
d87444 prot PYDNPVRTNQIPRQIPEQRWYMNRFVGILMAGILPFGAMFIELFFIFSAIWENQFYYLFGFL
Emp70 prot WDEHPTKTNQIARQIPFQPWYLKTIPATLIAGIFPFGSIAVELYFIYTSLWFNKIFYMFGFL
630 640 650 660 670 680
Contig# 1
SMBP prot MLVLVILCIVTVCVTIVCTYFLLNAEDYRWQWTSFLSAAST-AIYVYMYSFYYYFFKTKMYG
Arabidobsis prot LLVFVILVIVTVCVTIVGTYFLLNAENYHWQWTSFFSAAST-AVYVYLYSIYYYYVKTKMSG
hMP70 prot FFVFAILLSVGASISIALTYFQLSGEDYRWWWRSVLSVGST-GLFIFLYSVFYYARRSNMSG
p76 prot FLVFIILVITCSEATILLCYFHLCAEDYHWQWRSFLTSGFT-AVYFLIYAVHYFFSKLQITG
d87444 prot FLVFIILVVSCSQISIVMVYFQLCAEDYRWWWRNFLVSGGS-AFYVLVYAIFYFVNKLDIVE
Emp70 prot FFSFLLLTLTSSLVTILITYHSLCLENWKWQWRGFIIGGAGCALYVFIHSILF--TKFKLGG
690 700 710 720
Contig# 1
SMBP prot LFQTSFYFGYMAVFSTALGIMCGAIGYMGTSAFVRKIYTNVKID
Arabidobsis prot FFQTSFYFGYTMMFCLGLGILCGAVGYLGSNLFVRRIYFNIKCD
hMP70 prot AVQTVEFFGYSLLTGYVFFLMLGTISFFSSLKFIRYIYVNLKMD
p76 prot TASTILYFGYTMIMVLIFFLFTGTIGFFACFWFVTKIYSVVKVD
d87444 prot FIPSLLYFGYTALMVLSFWLLTGTIGFYAAYMFVRKIYAAVKID
Emp70 prot FTTIVLYVGYSSVISLLCCLVTGSIGFISSMLFVRKIYSSIKVD

FIGURE 22.2

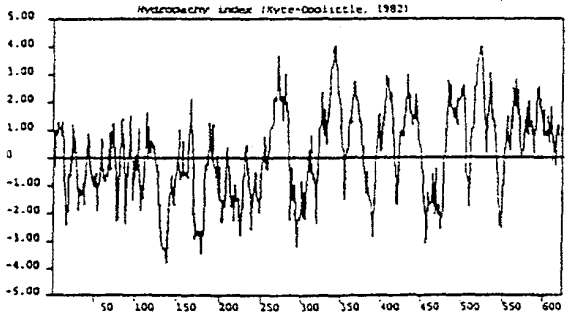
SMBP A



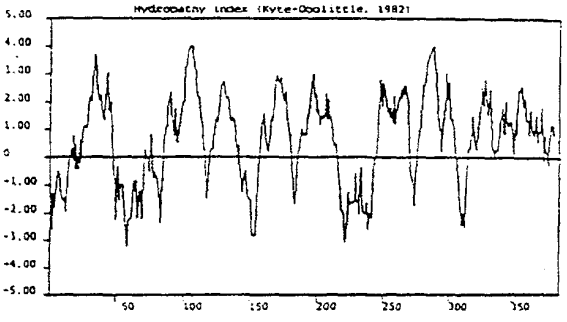
SMBP (C-ter) B



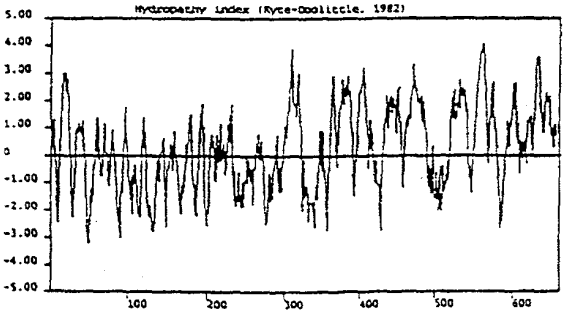
D87444



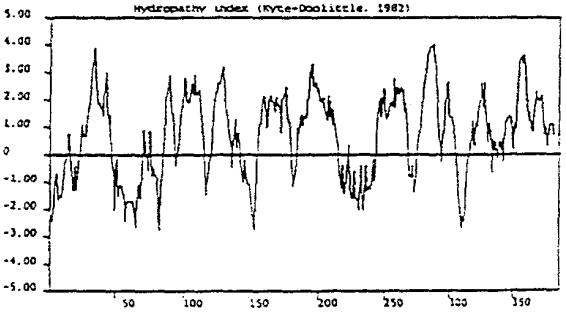
D87444 (C-ter)



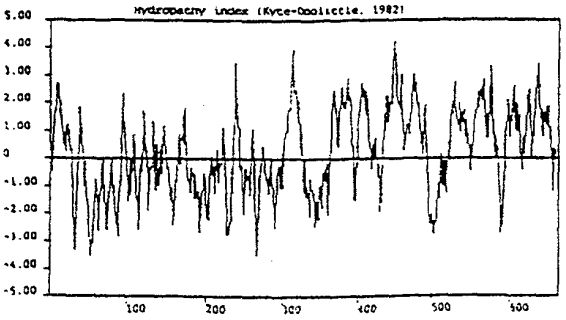
Hu p76



Hu p76 (C-ter)



Emp 70



Emp 70 (C-ter)

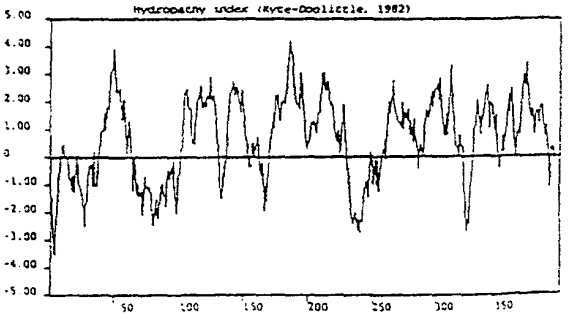
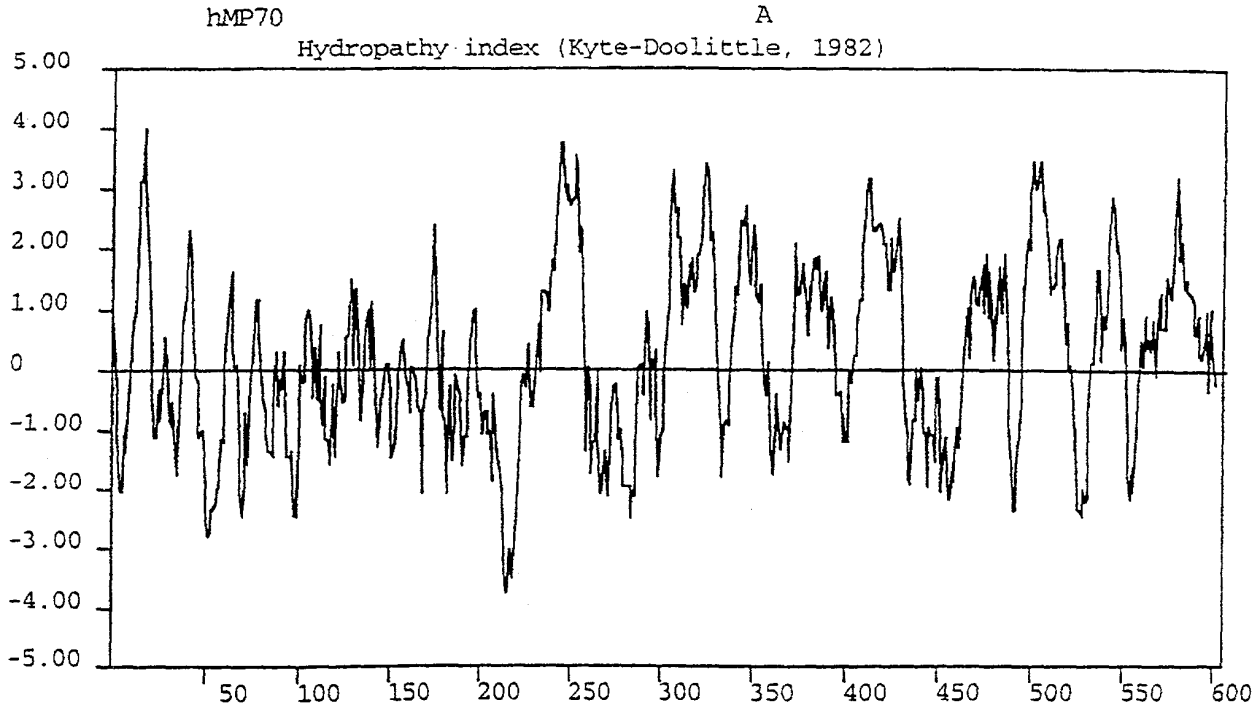


FIGURE 23.1



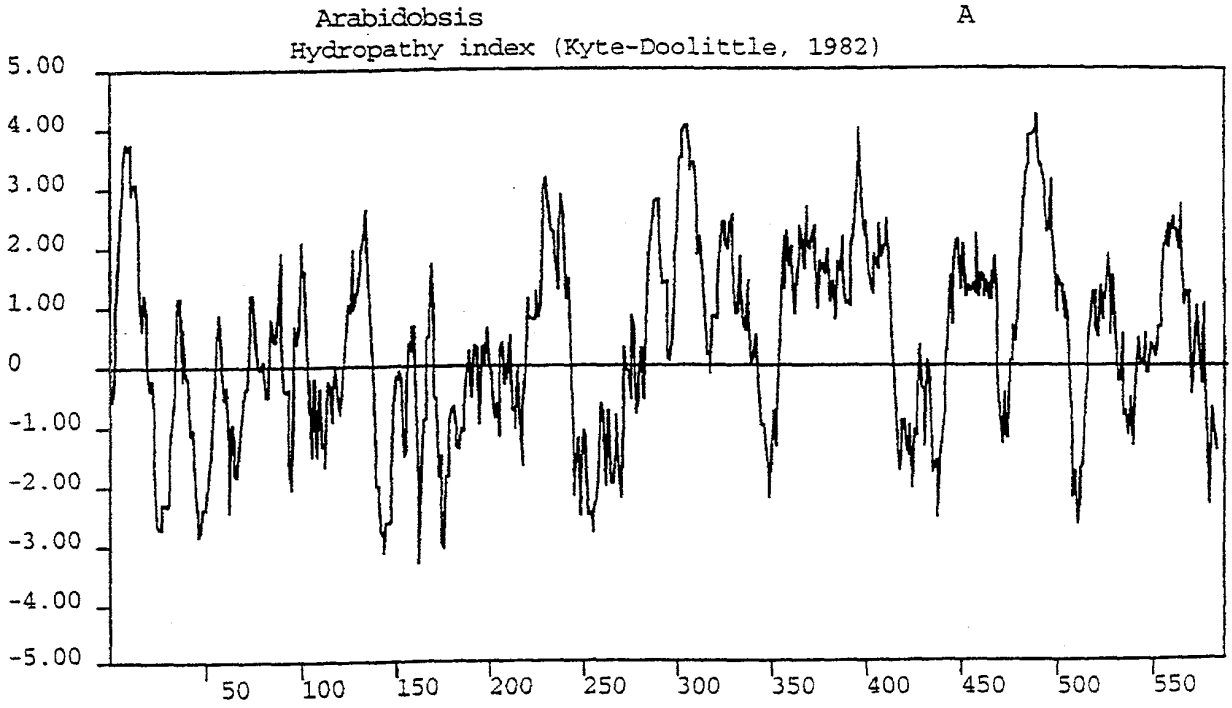


FIGURE 23.2

SUBSTITUTE SHEET (RULE 26)

A)

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1                                CC GCC GCG CTG TGG CTG CTG CTG CTG CTG CTG CCC CGG ACC CGG GCG GAC GAG CAC GAA CAC ACG TAT CAA GAT   74
1                                   A  A  L  W  L  L  L  L  L  L  P  R  T  R  A  D  E  H  E  H  T  Y  Q  D    24

75   AAA GAG GAA GTT GTC TTA TGG ATG AAT ACT GTT GGG CCC TAC CAT AAT CGT CAA GAA ACA TAT AAG TAC TTT TCA CTT CCA TTC TGT GTG  164
25   K  E  E  V  V  L  W  M  N  T  V  G  P  Y  H  N  R  Q  E  T  Y  K  Y  F  S  L  P  F  C  V    54

165  GGG TCA AAA AAA AGT ATC AGT CAT TAC CAT GAA ACT CTG GGA GAA GCA CTT CAA GGG GTT GAA TTG GAA TTT AGT GGT CTG GAT ATT AAA  254
55   G  S  K  K  S  I  S  H  Y  H  E  T  L  G  E  A  L  Q  G  V  E  L  E  F  S  G  L  D  I  K    84

255  TTT AAA GAT GAT GTG ATG CCA GCC ACT TAC TGT GAA ATT GAT TTA GAT AAA GAA AAG AGA GAT GCA TTT GTA TAT GCC ATA AAA AAT CAT  344
85   F  K  D  D  V  M  P  A  T  Y  C  E  I  D  L  D  K  E  K  R  D  A  F  V  Y  A  I  K  N  H    114

345  TAC TGG TAC CAG ATG TAC ATA GAT GAT TTA CCA ATA TGG GGT ATT GTT GGT GAG GCT GAT GAA AAT GGA GAA GAT TAC TAT CTT TGG ACC  434
115  Y  W  Y  Q  M  Y  I  D  D  L  P  I  W  G  I  V  G  E  A  D  E  N  G  E  D  Y  Y  L  W  T    144

435  TAT AAA AAA CTT GAA ATA GGT TTT AAT GGA AAT CGA ATT GTT GAT GTT AAT CTA ACT AGT GAA GGA AAG GTG AAA CTG GTT CCA AAT ACT  524
145  Y  K  K  L  E  I  G  F  N  G  N  R  I  V  D  V  N  L  T  S  E  G  K  V  K  L  V  P  N  T    174

525  AAA ATC CAG ATG TCA TAT TCA GTA AAA TGG AAA AAG TCA GAT GTG AAA TTT GAA GAT CGA TTT GAC AAA TAT CTT GAT CCG TCC TTT TTT  614
175  K  I  Q  M  S  Y  S  V  K  W  K  K  S  D  V  K  F  E  D  R  F  D  K  Y  L  D  P  S  F  F    204

615  CAA CAT CGG ATT CAT TGG TTT TCA ATT TTC AAC TCC TTC ATG ATG GTG ATC TTC TTG GTG GGC TTA GTT TCA ATG ATT TTA ATG AGA ACA  704
205  Q  H  R  I  H  W  F  S  I  F  N  S  F  M  M  V  I  F  L  V  G  L  V  S  M  I  L  M  R  T    234

705  TTA AGA AAA GAT TAT GCT CGG TAC AGT AAA GAG GAA GAA ATG GAT GAT ATG GAT AGA GAC CTA GGA GAT GAA TAT GGA TGG AAA CAG GTG  794
235  L  R  K  D  Y  A  R  Y  S  K  E  E  E  M  D  D  M  D  R  D  L  G  D  E  Y  G  W  K  Q  V    264

795  CAT GGA GAT GTA TTT AGA CCA TCA AGT CAC CCA CTG ATA TTT TCC TCT CTG ATT GGT TCT GGA TGT CAG ATA TTT GCT GTG TCT CTC ATC  884
265  H  G  D  V  F  R  P  S  S  H  P  L  I  F  S  S  L  I  G  S  G  C  Q  I  F  A  V  S  L  I    294

885  GTT ATT ATT GTT GCA ATG ATA GAA GAT TTA TAT ACT GAG AGG GGA TCA ATG CTC AGT ACA GCC ATA TTT GTC TAT GCT GCT ACG TCT CCA  974
295  V  I  I  V  A  M  I  E  D  L  Y  T  E  R  G  S  M  L  S  T  A  I  F  V  Y  A  A  T  S  P    324

975  GTG AAT GGT TAT TTT GGA GGA AGT CTG TAT GCT AGA CAA GGA GGA AGG AGA TGG ATA AAG CAG ATG TTT ATT GGG GCA TTC CTT ATC CCA  1064
325  V  N  G  Y  F  G  G  S  L  Y  A  R  Q  G  G  R  R  W  I  K  Q  M  F  I  G  A  F  L  I  P    354

1065 GCT ATG GTG TGT GGC ACT GCC TTC TTC ATC AAT TTC ATA GCC ATT TAT TAC CAT GCT TCA AGA GCC ATT CCT TTT GGA ACA ATG GTG GCC  1154
355  A  M  V  C  G  T  A  F  F  I  N  F  I  A  I  Y  Y  H  A  S  R  A  I  P  F  G  T  M  V  A    384

1155 GTT TGT TGC ATC TGT TTT TTT GTT ATT CTT CCT CTA AAT CTT GTT GGT ACA ATA CTT GGC CGA AAT CTG TCA GGT CAG CCC AAC TTT CCT  1244
385  V  C  C  I  C  F  F  V  I  L  P  L  N  L  V  G  T  I  L  G  R  N  L  S  G  Q  P  N  F  P    414

1245 TGT CGT GTC AAT GCT GTG CCT CGT CCT ATA CCG GAG AAA AAA TGG TTC ATG GAG CCT GCG GTT ATT GTT TGC CTG GGT GGA ATT TTA CCT  1334
415  C  R  V  N  A  V  P  R  P  I  P  E  K  K  W  F  M  E  P  A  V  I  V  C  L  G  G  I  L  P    444

1335 TTT GGT TCA ATC TTT ATT GAA ATG TAT TTC ATC TTC ACG TCT TTC TGG GCA TAT AAG ATC TAT TAT GTC TAT GGC TTC ATG ATG CTG GTG  1424
445  F  G  S  I  F  I  E  M  Y  F  I  F  T  S  F  W  A  Y  K  I  Y  Y  V  Y  G  F  M  M  L  V    474

1425 CTG GTT ATC CTG TGC ATT GTG ACT GTC TGT GTG ACT ATT GTG TGC ACA TAT TTT CTA CTA AAT GCA GAA GAT TAC CGG TGG CAA TGG ACA  1514
475  L  V  I  L  C  I  V  T  V  C  V  T  I  V  C  T  Y  F  L  L  N  A  E  D  Y  R  W  Q  W  T    504

1515 AGT TTT CTC TCT GCT GCA TCA ACT GCA ATC TAT GTT TAC ATG TAT TCC TTT TAC TAC TAT TTT TTC AAA ACA AAG ATG TAT GGC TTA TTT  1604
505  S  F  L  S  A  A  S  T  A  I  Y  V  Y  M  Y  S  F  Y  Y  Y  F  F  K  T  K  M  Y  G  L  F    534

1605 CAA ACA TCA TTT TAC TTT GGA TAT ATG GCG GTA TTT AGC ACA GCC TTG GGG ATA ATG TGT GGA GCG ATT GGT TAC ATG GGA ACA AGT GCC  1694
535  Q  T  S  F  Y  F  G  Y  M  A  V  F  S  T  A  L  G  I  M  C  G  A  I  G  Y  M  G  T  S  A    564

1695 TTT GTC CGA AAA ATC TAT ACT AAT GTG AAA ATT GAC TAG AGACCCAAGAAAACCTGGAACTTTGGATCAATTTCTTTTTCATAGGGGTGGAACTTGCACAGCAAAA  1800
565  F  V  R  K  I  Y  T  N  V  K  I  D  *                                                                                  576
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B)

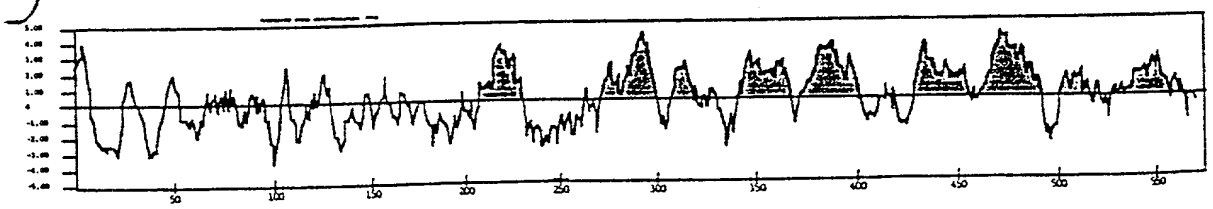

FIGURE 24

SUBSTITUTE SHEET (RULE 26)

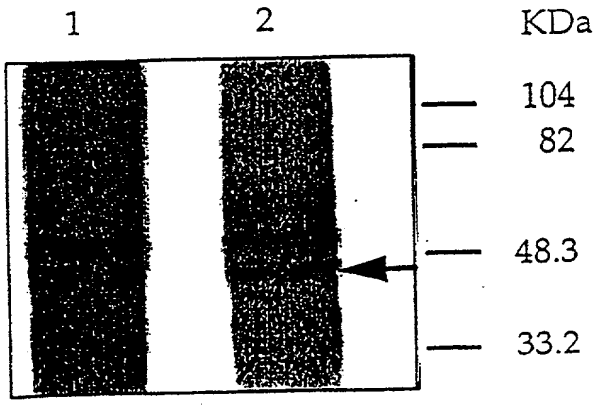

FIGURE 25

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office

ATTORNEY DOCKET NO.: 045636-5025

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

MAMMALIAM ICYP (IODOCYANOPINDOLOL) RECEPTOR AND ITS APPLICATIONS

the specification of which:

is attached hereto; or

was filed as United States application Serial No. 09/319,724 on June 11, 1999 and was amended on _____ (if applicable); or

was filed as PCT international application Number PCT/EP97/07339 on December 12, 1997 and was amended under PCT Article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office information which is material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN APPLICATION(S):

COUNTRY (if PCT, indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
EP	96/402719.7	12 December 1996	[X] Yes [ ] No
			[ ] Yes [ ] No
			[ ] Yes [ ] No
			[ ] Yes [ ] No

Combined Declaration For Patent Application and Power of Attorney - **(Continued)**
(includes Reference to PCT International Applications)

ATTORNEY DOCKET NO.: 045636-5023

I hereby claim the benefits under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

U.S. PROVISIONAL APPLICATIONS

U.S. PROVISIONAL APPLICATION NO.	U.S. FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or §365(c) of any PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT:

U.S. APPLICATIONS		STATUS (Check One)		
U.S. APPLICATION NO.	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint the registered practitioners of Morgan, Lewis & Bockius LLP included in the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number.

Customer Number: 009629

Direct Telephone Calls To:
(name and telephone number)

Reid G. Adler
202-467-7756

Combined Declaration For Patent Application and Power of Attorney - **(Continued)**
(includes Reference to PCT International Applications)

ATTORNEY DOCKET NO.: 045636-5025

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF SOLE OR FIRST INVENTOR	**Gerlinde LENZEN**	
RESIDENCE & CITIZENSHIP	Paris, France FR	COUNTRY OF CITIZENSHIP FRANCE
POST OFFICE ADDRESS	55, rue des Cévennes, 75015 Paris, France	
FIRST OR SOLE INVENTOR'S SIGNATURE	Gerlinde Lenzen	DATE August 10, 1999
FULL NAME OF SECOND INVENTOR	**Arthur Donny STROSBERG**	
RESIDENCE & CITIZENSHIP	Paris, France FR	COUNTRY OF CITIZENSHIP FRANCE
POST OFFICE ADDRESS	66, rue de Javel, 75015 Paris, France	
SECOND INVENTOR'S SIGNATURE	Arthur Donny Strosberg	DATE August 10, 1999
FULL NAME OF THIRD INVENTOR	**Toshinari SUGASAWA**	
RESIDENCE & CITIZENSHIP	Osaka, Japan JP	COUNTRY OF CITIZENSHIP JAPAN
POST OFFICE ADDRESS	9-12-507 Miyano-cho, Takatsuki-shi, Osaka 569, France	
THIRD INVENTOR'S SIGNATURE	菅沢敏成	DATE Aug 10, 1999

Listing of Inventors Continued on attached page(s) [X] Yes [ ] No

Combined Declaration For Patent Application and Power of Attorney - **(Continued)**
(includes Reference to PCT International Applications)

ATTORNEY DOCKET NO.: 045636-5025

FULL NAME OF FOURTH INVENTOR	**Shigeaki MOROOKA**	
RESIDENCE & CITIZENSHIP	Hyogo, Japan JPX	COUNTRY OF CITIZENSHIP JAPAN
POST OFFICE ADDRESS	4-78 nishi-3, Seiwadai, Kawanishi-shi, Hyogo, 666-01 Japan	
FOURTH INVENTOR'S SIGNATURE	諸岡茂昭	DATE Aug. 9. 1999
FULL NAME OF FIFTH INVENTOR		
RESIDENCE & CITIZENSHIP		COUNTRY OF CITIZENSHIP
POST OFFICE ADDRESS		
FIFTH INVENTOR'S SIGNATURE		DATE
FULL NAME OF SIXTH INVENTOR		
RESIDENCE & CITIZENSHIP		COUNTRY OF CITIZENSHIP
POST OFFICE ADDRESS		
SIXTH INVENTOR'S SIGNATURE		DATE
FULL NAME OF SEVENTH INVENTOR		
RESIDENCE & CITIZENSHIP		COUNTRY OF CITIZENSHIP
POST OFFICE ADDRESS		
SEVENTH INVENTOR'S SIGNATURE		DATE

Listing of Inventors Continued on attached page(s) [ ] Yes [X] No

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